AD_____

Award Number: DAMD17-03-1-0013

TITLE: Rescuing High Avidity T Cells for Prostate Cancer Immunotherapy

PRINCIPAL INVESTIGATOR: Pan Zheng, M.D., Ph.D.

CONTRACTING ORGANIZATION: Ohio State University
Columbus, OH 43210-1239

REPORT DATE: April 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| 01-04-2006 | Final | 1 Apr 2003 – 31 Mar 2006 |

**4. TITLE AND SUBTITLE**

Rescuing High Avidity T Cells for Prostate Cancer Immunotherapy

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
DAMD17-03-1-0013

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

Pan Zheng, M.D., Ph.D.

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Ohio State University
Columbus, OH 43210-1239

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**
Original contains colored plates: ALL DTIC reproductions will be in black and white.

**14. ABSTRACT** This is the final report on the grant "Rescuing high avidity T cells for prostate cancer immunotherapy". The purpose of the grant proposal is to rescuing high avidity tumor-antigen specific T cells that can respond effectively to prostate cancer cells and delay the development of prostate cancer in the TRAMP mouse model. We have proposed three specific aims. (1). Identify the cells in thymus that express peripheral tumor antigen to induce clonal deletion of tumor antigen reactive T cells. (2). Examine whether anti-B7 antibody treatment in TRAMP mice can rescue the tumor-antigen specific T cells that are otherwise deleted. (3). Determine the thymic function in prostate cancer patients undergoing hormonal therapy. In the past funding period, we have published two papers that identifying specific cell types in thymus that induce clonal deletion of tumor antigen reactive T cells and elucidating the role of costimulatory molecule B7 on T cell early development. We had one paper under revision on the role of B7 on NKT cell development and another manuscript in preparation on the role of B7 in regulatory T cell development. We have generated preliminary data on the treatment with lymphotoxin b receptor fusion protein to rescue the high avidity tumor antigen specific T cells. We have shown that anti-B7 treatment changed the regulatory T cell numbers and increased the survival time of TRAMP mice.

**15. SUBJECT TERMS**
Immune interactions, immunotherapy, T cell development, tumor progression.

| 16. SECURITY CLASSIFICATION OF: | | | | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| | | | | | USAMRMC |
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | 19b. TELEPHONE NUMBER *(include area code)* |
| U | U | U | UU | 67 | |

## Table of Contents

## (4) Introduction

Most tumor antigens have the same sequences as the endogenous genes. In addition to their over-expression in the tumors, essentially all of these antigens are expressed in some normal tissues, which is analogous to the so-called peripheral antigen. Most recent studies showed that many of the peripheral antigens are expressed in specialized cells in the thymus, and can induce central tolerance of their specific T cells. However, it is not clear whether mechanisms responsible for tolerizing peripheral antigen in the thymus are responsible for tolerizing potential cancer-specific T cells. We have recently taken a double-transgenic approach to study the mechanism of immune tolerance to a transgenic antigen, SV40-large T antigen (Tag), in the transgenic adenocarcinoma in mouse prostate (TRAMP) mice. We have showed that central tolerance plays an important role in immune tolerance to TRAMP mice. In addition, we have published strong evidence that blockage of T cell costimulatory pathway can break the central tolerance for a large array of autoreactive T cells**.** In this proposal, we have hypothesized that blockade of the T cell costimulatory pathway in adults would inhibit the deletion of high avidity tumor antigen specific T cells. The rescued high avidity tumor-antigen specific T cells can respond effectively to prostate cancer cells and delay the development of prostate cancer in the TRAMP model. We proposed to carry out the following specific aims. (1). Identify the cells in thymus that express peripheral tumor antigen to induce clonal deletion of tumor antigen reactive T cells. (2). Examine whether anti-B7 antibody treatment in TRAMP mice can rescue the tumor-antigen specific T cells that are otherwise deleted. (3). Determine the thymic function in prostate cancer patients undergoing hormonal therapy.

During the first year, we have completed the studies outlined in specific aim 1. We have generated the bone marrow (BM) radiation chimera mice to study the existence, contribution and mechanism of the hematopoietic peripheral antigen expressing (PAE) cells in tolerance to tissue-specific antigens. Our results revealed that BM-derived PAE exist in both central and secondary lymphoid organs and that the expression of peripheral antigens in the BM-derived cells does not correlate with *aire* expression. Using double transgenic mice expressing TCR specific for a model antigen expressed under the control of the prostate-specific promoter, we show that expression of the self antigen in PAE of non-hematopoietic origin is both necessary and sufficient to induce clonal deletion. Surprisingly, while BM-derived PAE failed to induce clonal deletion, they did cause the activation-induced cell death of autoreactive cells in the secondary lymphoid organs. Thus, the BM-derived PAE have a distinct function in the maintenance of tolerance to tissue-specific antigens.

There were two manuscripts published in Eur. J. Immunol. and J. Immunol. in second year. During the process of completing the specific aim 1 and 2, we extended our study in the role of costimulatory molecule in NKT cell development. Natural Killer T (NKT) cell is a unique subset of T cells characterized by expressing both invariant TCR and various NK linage markers. NKT cells are actively involved in viral infection, autoimmunity and tumor immunity. NKT cell developed in thymus and deviated from mainstream of conventional T cell development at CD4+CD8+ double positive stage. We showed that the development of NKT cells are defect in the mice with targeted mutations of B7-1/2 and CD28, the percentage of $TCR_{\beta}^{+}NK1.1^{+}$, as well as $TCR_{\beta}^{+}$ α-Galcer/$CD1d^{+}$ (iVα14 NKT) cells population are significantly reduced in the thymus, spleen and liver. In consistent with these results, the mice with target mutation of costimulatory molecules

has defect NKT cell function. B7 and CD28 deficient mice develop much less sever ConA induced hepatitis, which is known mediated by NKT cells. Taken together, our results demonstrate that development and function of NKT cell is subject to modulation by the costimulatory pathway.

We made significant progress in specific aim 2 in the third year. We discovered that the effect of prolonged survival in TRAMP mice after administration of anti-B7 antibodies may due to the rapid disappearance of regulatory T cells in the peripheral lymphoid organs. We further studied the role of B7 in the Treg development and peripheral regulation.

The observations made in the past funding period and the collaboration between my laboratory and Dr. Yang-Xin Fu from University of Chicago have identified cell-surface interactions that are critical for clonal deletion and generation of Treg. These preliminary results formed the experimental foundation of a new proposal (PC051274, score: 2.1, resubmitted as PC061244) that has been submitted to DAMD to support the hypothesis that it is possible to reset the T cell repertoire in the cancer-bearing host, mostly using reagents that can be easily adapted to immune therapy in cancer.

## (5) Body of Annual Report

*Task 1.* *What are the cells in thymus that express tumor-associated antigen that induce clonal deletion of potentially tumor antigen reactive T cells? (Month 1-18).* (Completed).

*a. To breed TRAMP mice with TCR transgenic mice TG-B to produce 4 different types of F1 mice: WT F1 and $Tag^+/TCR^-$ F1 (to be used as recipient mice); $Tag^-/TCR^+$ F1 and $Tag^+/TCR^+$ F1 (to be used as bone marrow donor mice). 10-15 F1 mice for each group are needed. (Months 1-12).* (Completed).

*b. To perform the bone marrow irradiation chimera experiments. Each animal experiment cycle requires 12 weeks. Three independent experiments will be performed. Average 5 mice per group with 4 groups in each experiment. (Months 4-15).* (Completed).

*c. To perform in vitro experiments, such as immunofluorescent study, Immunohistochemical studies, and lymphocyte functional assays (Months 13-18).* (Completed).

*d. To perform the modified bone marrow irradiation chimera experiment involving surviving surgery (thymectomy and thymus engrafting). Each animal experiment cycle requires 16 weeks. Three independent experiments will be performed. Average 5 mice per group with 4 groups in each experiment (Months 7-18).* (These were alternative experiments to compliment the Task 1-b and 1-c. Since the bone marrow chimera experiments gave us conclusive results, it is not necessary for us to perform Task 1-d).

We have completed the Task 1 and the results have been summarized in the manuscript "Expression of tissue-specific autoantigens in the hematopoietic cells leads to activation-induced cell death of autoreactive T cells in the secondary lymphoid organs" that published in EJI and attached as Appendix 1.

During the process, we had made some unexpected observations related to the function of costimulatory molecules in early stages of T cell development. The manuscript "B7-CD28 Interaction Promotes Proliferation and Survival but Suppresses Differentiation of $CD4^-CD8^-$ T Cells in the Thymus" is published in the Journal of Immunology and attached as Appendix 2.

We also extended our study on the role of costimulatory molecules in NKT cell development. The manuscript is at the final stage of preparation and we intend to submit to Journal of Immunology. The manuscript is attached as Appendix 3.

***Task 2.*** ***Will anti-B7 antibody treatment in adult animal rescue the tumor-antigen specific T cells that are otherwise deleted? (Month 1-36).*** **(Completed).**

*a. To test the effect of anti-B7 antibody treatment on the clonal deletion of SV40 Tag specific transgenic T cells in F1 $Tag^+/TCR^+$ double transgenic mice. Each animal experiment cycle requires 16 weeks. Average 5 mice per group with 2 groups in each experiment. 4 different starting ages will be used. Three independent experiments for each age will be performed. (Months 1-18). (Completed).*

*b. Test the effect of anti-B7 antibody treatment on the TRAMP mice with polyclonal T cell repertoire. Each animal experiment cycle requires 16 weeks. Average 5 mice per group with 2 groups in each experiment. 4 different*

*starting ages will be used. Three independent experiments for each age will be performed. (Months 7-24). (Completed).*

*c. We will observe the tumor development in TRAMP mice to test whether the transient block of T cell development by anti-B7 antibody treatment can delay tumor progression. (Months 7-36). (Completed).*

*d. We will determine the time window that optimizes the effect of anti-B7 antibody treatment (Months 25-36). (Completed).*

We have carried out the experiments outlined in specific aim 2. We have found that B7-1 and B7-2 are required for the production of regulatory T cells and anti-B7 antibody treatment prolongs the survival time of TRAMP mice.

We have compared the number of $CD4^+CD25^+$ Treg in the thymus and spleen of WT and B7-deficient mice. As shown in Fig. 1, targeted mutation of B7-1/2 leads to more than 6-fold reduction in the percentage of Treg in both thymus and spleen. Similar reductions in the central and peripheral tissues suggest that the reduction is primarily due to the developmental defect of Treg, although a contribution of costimulation in homeostasis and survival of Treg, as proposed by others, is also possible. To test if the number of Treg can be modulated by anti-B7-1 and anti-B7-2 antibodies, we injected the two antibodies into C57BL/6 and B6 TRAMP mice. As shown in Fig. 2, the anti-B7 antibodies significantly reduced the number of Treg in the thymus and in the spleen.

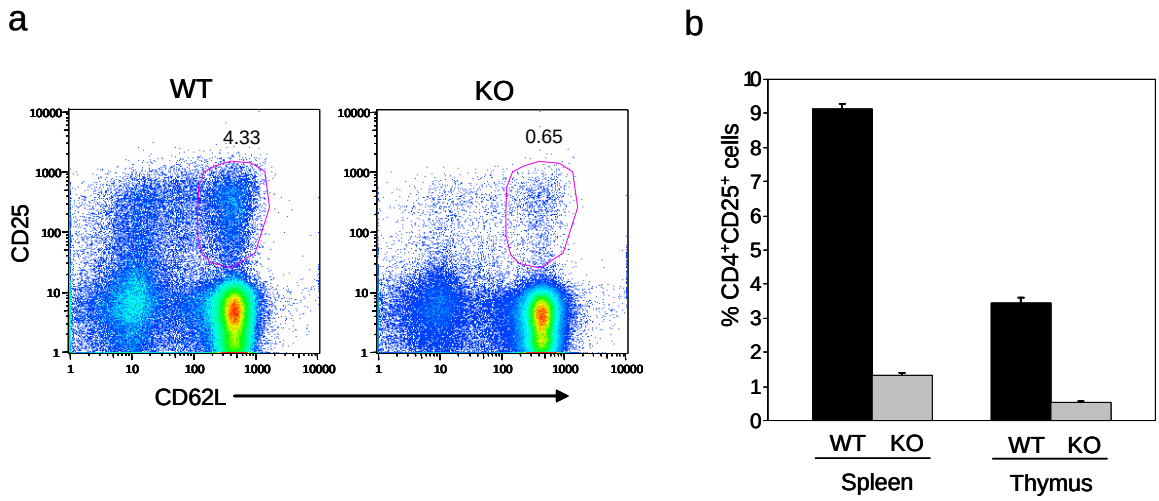


**Fig. 1.** B7-deficient mice have reduced numbers of $CD4^+CD25^+$ regulatory T cells in the spleen and thymus. Spleens and thymi from 5-6 week old female WT and B7-deficient mice were harvested and stained for the presence of regulatory T cells. a) Representative FACS plot of $CD25^+CD62L^+$ cells within the $CD4^+$ small spleen cell gate from wild-type and B7(-/-) mice. b) Columns represent mean percentage of $CD4^+CD25^+$ cells within gated small cells in spleens or thymi of 4 mice per group. Error bars represent SEM.

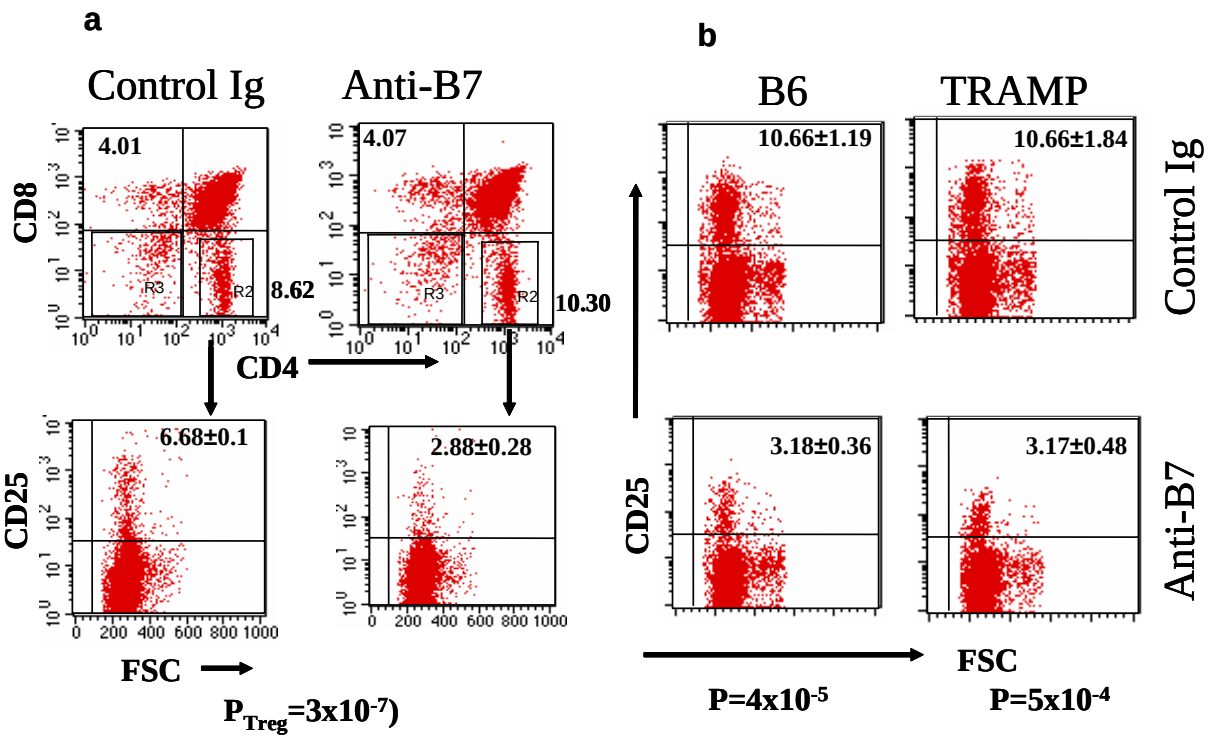


**Fig. 2**. Anti-B7-1 and anti-B7-2 treatment blocks development of Treg in the thymus (a) and accumulation of Treg in the spleen. The mice (WT B6 and TRAMP mice, 4 of each group) were treated with either anti B7-1(GL-1)/B7-2(3A12) or Control Ig (rat and hamster IgG, 100 μg/injection) every other day for 6 treatments. The mice were sacrificed 8 days after last treatment and analyzed by 4-color flow cytometry. A). Data shown are profiles of B6 thymocytes with total thymocytes on top and gated CD4 T cells at the bottom. Similar results were observed in the TRAMP mice. B). Profiles of gated CD4 T cells from spleens of B6 and TRAMP mice. These results are representatives of 2 independent experiments.

To determine the impact of transient Treg reduction in the TRAMP model, we injected 4 week old mice with anti-B7-1 and anti-B7-2 (6 injections over 2 weeks) and followed the mice over a one year period for their survival. As shown in Fig. 3, a short-term treatment with anti-B7-1 and anti-B7-2 substantially reduced the rate of prostate cancer death in the TRAMP mice.

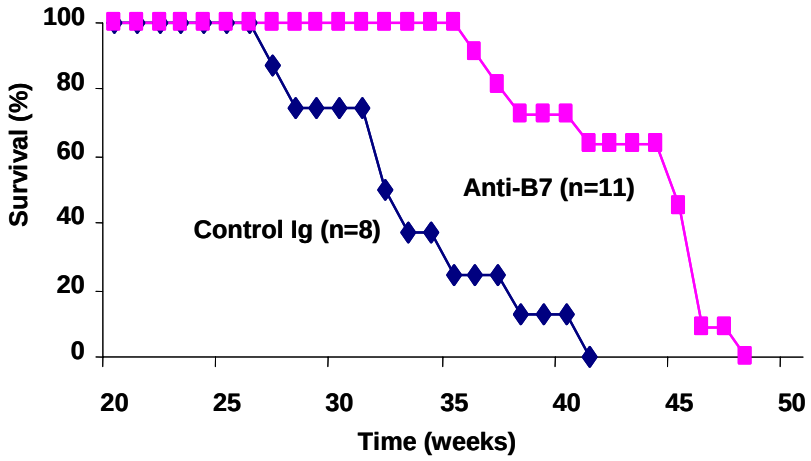


**Fig. 3**. Anti-B7-treatment of TRAMP mice increases the overall survival. Young TRAMP mice were treated with either anti-B7-1/anti-B7-2 or control IgG every other day for 6 treatments, and were observed over a 50 week period. The endpoint of the experiment is the prostate cancer reaching approximately 2 cm in diameter. $P<0.001$.

Meanwhile, we have generated preliminary data that showed that *Aire*-deficiency and soluble LTβRIg can block the expression of prostate-specific genes in the thymus and modulates clonal deletion of tumor-reactive T cells.

Recent studies revealed *aire* as a master regulator for expression of many tissue-specific antigens in the thymus. In order to determine whether *aire* also regulates expression of prostate-specific genes in the thymus, we compared the expression of probasin along with other tissue specific genes in the thymus from wild type (WT) and *aire*-deficient mice. As shown in Fig. 4, targeted mutation of *aire* gene caused a 200-fold reduction in expression of probasin gene in the thymus. Thus, expression of at least one of the prostate-specific genes is under the control of *aire*. Since the probasin promoter is used in the TRAMP mice, it is likely that *aire* also controls expression of the model tumor antigen Tag in the TRAMP model.

Our collaborator, Dr. Yang-Xin Fu, has demonstrated lymphotoxin (LT) controls expression of *aire* and therefore controls the expression of tissue-specific antigens in the thymus. In addition, administration of LTβRIg can interfere with expression of the tissue specific antigens. To determine if LTβRIg can be used to modulate the expression of tumor antigen and clonal deletion of T cells in the TRAMP mice model, we treated the TRAMPxTgB F1 mice with LTβRIg or control IgG three times with 3-day intervals and analyzed the expression of Tag. As shown in Fig. 5a, relative to control IgG group, LTβRIg reduced expression of Tag by almost 7-fold. More importantly, LTβRIg drastically reduced the deletion of the $CD4^+CD8^+$ T cells in the thymus (Fig. 5b). These preliminary studies raised the possibility that LTβRIg can be used to rescue tumor-reactive T cells.

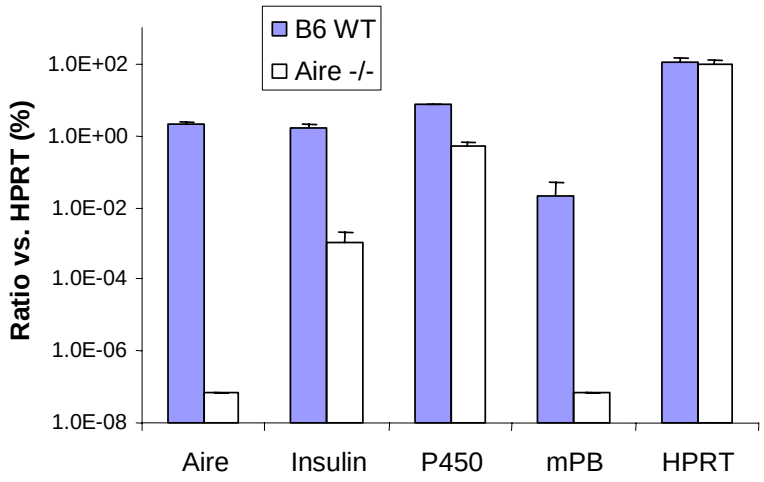


**Fig. 4**. Targeted mutation of *aire* abrogates expression of probasin gene in the thymus. Thymus tissues from WT and *aire*-deficient mice were homogenized and analyzed for the expression of several tissue-specific genes. Data shown are means and S.D. of triplicates and represent two independent experiments.

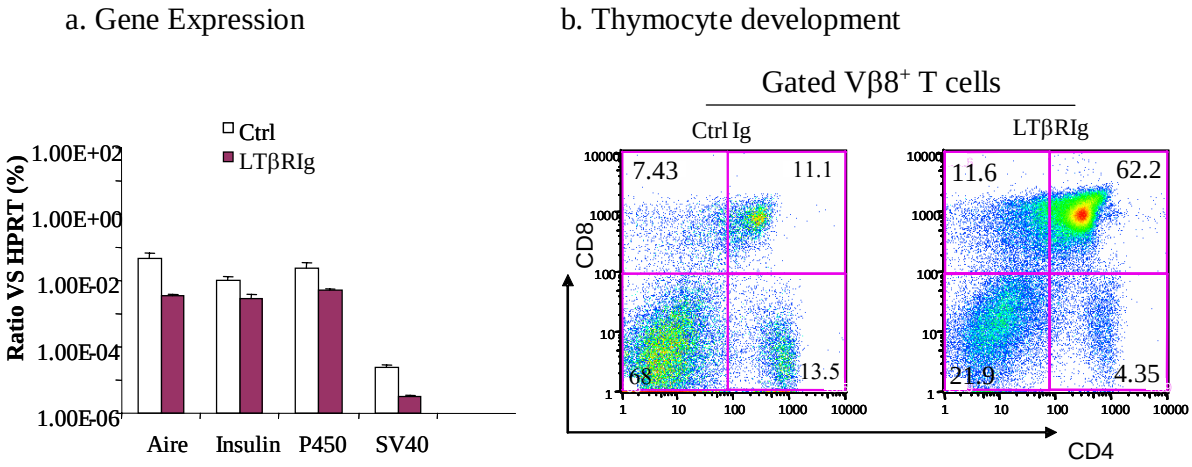


**Fig. 5.** Treatment with soluble LTβRIg blocks expression of tumor antigen in the thymus and prevents clonal deletion of tumor-reactive T cells. TRAMP/TgB double transgenic mice received 3 treatments of either control IgG or LTβRIg at 3-day intervals. At day 9 after the first treatment, the thymi were harvested. Half of the thymic tissue were used to prepare mRNA to analyze expression of tumor antigen and other tissue-specific genes, while the other half were used to prepare single-cell suspension of thymocytes for flow cytometry. a. Gene expression as determined by real-time PCR. b. Distribution of thymocyte subsets in control Ig and LTβRIg-treated mice. Data shown are one representative from each group of two mice. Note a substantial increase of $CD4^+CD8^+$ thymocytes and an approximately 50% increase of $CD8^+CD4^-$ T cells.

***Task 3. To examine the thymic function in human prostate cancer patients (Months 1-36).*** **(Encounter difficulty; applied for suspension of Task 3 in Annual Report 2005).**

*a. Obtain prostate cancer patients' consents and collect clinical information (Month 1-36).*

*b. Coordinate with urologists and establish schedule to collect blood samples from prostate cancer patients undergo different therapy modules (Month 4-36).*

*c. Establish reliable experimental procedure to isolate different subsets of lymphocytes from peripheral blood mononuclear cell fractions. Identify reliable PCR condition to detect the levels of TRECs from T lymphocytes. To set up real-time PCR and QC-PCR protocol to get good reproducibility. (Month 1-6).*

*d. To perform QC-PCR to detect TRECs from lymphocytes from prostate cancer patients. (Month 7-36).*

*e. To perform statistical analysis on data (Month 25-36).*

Taken together, we have established that expression of tissue-specific antigen/tumor antigen in the thymus and clonal deletion of tumor-reactive T cells can be modulated in the transgenic mouse model of prostate cancer (TRAMP). Likewise, we showed that the development of Treg in the thymus depends on costimulatory molecules and can be abrogated by treatment with anti-B7 mAbs. Our results demonstrate that the understanding of the development of both tumor-reactive and Treg can provide a novel approach for tumor immunotherapy.

## (6) Key Research Accomplishments

- We have generated the bone marrow (BM) radiation chimera mice to study the existence, contribution and mechanism of the hematopoietic peripheral antigen expressing (PAE) cells in tolerance to tissue-specific antigens.
- We have shown that BM-derived PAE exist in both central and secondary lymphoid organs.
- We have examined the expression of peripheral antigens in the BM-derived cells and demonstrated that its expression does not correlate with *aire* expression.
- Using double transgenic mice expressing TCR specific for a model antigen expressed under the control of the prostate-specific promoter, we have shown that expression of the self antigen in PAE of non-hematopoietic origin is both necessary and sufficient to induce clonal deletion.
- We have demonstrated that while BM-derived PAE failed to induce clonal deletion, they caused the activation-induced cell death of autoreactive cells in the secondary lymphoid organs. Thus, the BM-derived PAE have a distinct function in the maintenance of tolerance to tissue-specific antigens.
- We showed that the development of NKT cells are defect in the mice with targeted mutations of B7-1/2 and CD28. The percentage of $TCR_{\beta}^{+}NK1.1^{+}$, as well as $TCR_{\beta}^{+}$ α-Galcer/$CD1d^{+}$ (iVα14 NKT) cells population are significantly reduced in the thymus, spleen and liver in the mice with targeted mutations of B7-1/2 and CD28.
- We have shown the mice with target mutation of costimulatory molecules has defect NKT cell function. B7 and CD28 deficient mice develop much less sever ConA induced hepatitis, which is known mediated by NKT cells.
- We have found that B7-1 and B7-2 are required for the production of regulatory T cells. Anti-B7 antibodies significantly reduced the number of Treg in the thymus and in the spleen.
- We have shown that anti-B7 antibody treatment in adult TRAMP mice prolonged the mice survival for more than 10 weeks.
- We have established that expression of tissue-specific antigen/tumor antigen in the thymus and clonal deletion of tumor-reactive T cells can be modulated in the TRAMP mice.

**(7) Reportable Outcomes:**
**Publications directly funded by DAMD17-03-1-0013.**

1. Xincheng Zheng, Lijie Yin, Yang Liu and Pan Zheng. 2004. Hematopoietic peripheral antigen expressing cells impose activation-induced cell death of autoreactive T cells in the secondary lymphoid organs. *Eur. J. Immunol.* 34:3126-34. (Appendix 1).

2. Xincheng Zheng, Jian-Xin Gao, Xing Chang, Yin Wang, Yan Liu, Jin Wen, Jian Jiang, Yang Liu and Pan Zheng. 2004. B7-CD28 Interaction Promotes Proliferation and Survival but Suppresses Differentiation of $CD4^-CD8^-$ T Cells in the Thymus. *J. Immunol.* 173:2253-61. (Appendix 2).

3. Xincheng Zheng, Yang Liu and Pan Zheng. 2006. The B7-CD28 interaction is critical in the development and effector function of NKT cells. (Appendix 3).

**Degree awarded:**

Xincheng Zheng has been award Ph.D. degree by the Graduate School of The Ohio State University on Mar. 22, 2005.

**Funding applied:**

PC051274, Resetting the T cell repertoire in prostate cancer bearing host. Score: 2.1.
Innovation: 8.3
Research Strategy: 7.2
Disease relevance: 4.6
Personnel: 8.7
Environment: 8.7

PC061244, Resetting the T cell repertoire in prostate cancer bearing host. Submitted in April, 2006.

**Personnel: all from Division of Cancer Immunology, Dept of Pathology, OSU.**

Pan Zheng, MD, PhD, Principal Investigator. 25% effort, from 4/1/03 to 4/30/06.
Xincheng Zheng, PhD, Graduate Research Assistant. 100% effort, from 4/1/03 to 4/8/05.
Penghui Zhou, Graduate Research Assistant, 100% effort, from 6/15/04 to 4/30/06.
Runhua Liu, PhD, Post-doctoral Associate, 50% effort, from 4/1/05 to 4/30/06.
Huiming Zhang, Research Associate, 50% effort, from 4/1/03 to 4/1/05.

## (8) Conclusions:

In summary, we have made important progress during the three year funding period. We have followed the Statement of Work closely. We have completed the Task 1 and Task 2. Taken together, we have established that expression of tissue-specific antigen/tumor antigen in the thymus and clonal deletion of tumor-reactive T cells can be modulated in the transgenic mouse model of prostate cancer (TRAMP). Likewise, we showed that the development of Treg in the thymus depends on costimulatory molecules and can be abrogated by treatment with anti-B7 mAbs. Our results demonstrate that the understanding of the development of both tumor-reactive and Treg can provide a novel approach for tumor immunotherapy.

Our progress is fundamentally important for future development of new ways for prostate cancer immunotherapy. Various ideas and methods have been developed to augment anti-tumor immunity. Most of the therapies aim at activation of T cells that are already present in peripheral T cell repertoire. However, our preliminary data provide definitive evidence that high avidity tumor antigen specific T cells are deleted through central tolerance. As such, the existing approaches aim at expanding what is likely to be low avidity tumor reactive T cells. This proposal will explore an innovative idea that high avidity tumor antigen specific T cells can be rescued from clonal deletion by blockade of T cell costimulatory pathway. We anticipate that the rescued T cells will be much more powerful in the combat against tumor. The relevance of our study is further increased by two important factors. First, utilization of spontaneous prostate cancer model will allow us to build a solid experimental foundation for a novel immunotherapy of prostate cancer. Second, the widely used hormonal therapy of prostate-cancer patients may create a new wave of T cell thymopoiesis among them. The random gene re-arrangement of TCR gene during the process may provide a new source of high-avidity prostate cancer-specific T cells to be rescued by our novel approach.

Due to the changes in the Division of Urological Surgery in OSU, we encountered difficulty in enrolling prostate cancer patients in our Specific Aim 3 study. We have applied to suspend the Task 3. However, we were very excited to read from current literature on similar studies that we proposed to do. Hormone (androgen ablation) therapy has become a standard palliative treatment for patients with advanced prostate cancer. Studies have shown that androgen ablation therapy increases the levels of circulating lymphocytes(1) and induces prominent T cell infiltration to prostate cancerous tissue(2). More recent works by Dr. Richard Boyd's group provided strongest evidence that androgen ablation results in the complete regeneration of the aged male mouse thymus, restoration of peripheral T cell phenotype and functions and enhanced thymus regeneration following bone marrow transplantation(3-5). These seminal works greatly increase the clinical relevance of our work in human prostate cancer patients.

## (9) References:

1. Oliver, R. T., and Gallagher, C. J. Intermittent endocrine therapy and its potential for chemoprevention of prostate cancer. Cancer Surv *23:*191-207. 1995.
2. Mercader, M., Bodner, B. K., Moser, M. T., Kwon, P. S., Park, E. S., Manecke, R. G., Ellis, T. M., Wojcik, E. M., Yang, D., Flanigan, R. C., Waters, W. B., Kast, W. M., and Kwon, E. D. T cell infiltration of the prostate induced by androgen withdrawal in patients with prostate cancer. Proc Natl Acad Sci U S A *98:*14565-70. 2001.
3. Goldberg, G. L., Sutherland, J. S., Hammet, M. V., Milton, M. K., Heng, T. S., Chidgey, A. P., and Boyd, R. L. Sex steroid ablation enhances lymphoid recovery following autologous hematopoietic stem cell transplantation. Transplantation *80:*1604-13. 2005.
4. Heng, T. S., Goldberg, G. L., Gray, D. H., Sutherland, J. S., Chidgey, A. P., and Boyd, R. L. Effects of castration on thymocyte development in two different models of thymic involution. J Immunol *175:*2982-93. 2005.
5. Sutherland, J. S., Goldberg, G. L., Hammett, M. V., Uldrich, A. P., Berzins, S. P., Heng, T. S., Blazar, B. R., Millar, J. L., Malin, M. A., Chidgey, A. P., and Boyd, R. L. Activation of thymic regeneration in mice and humans following androgen blockade. J Immunol *175:*2741-53. 2005.

# Expression of tissue-specific autoantigens in the hematopoietic cells leads to activation-induced cell death of autoreactive T cells in the secondary lymphoid organs

**Xincheng Zheng, Lijie Yin, Yang Liu and Pan Zheng**

Division of Cancer Immunology, Department of Pathology and Comprehensive Cancer Center, The Ohio State University, Columbus, USA

Many tissue-specific antigens are expressed in specialized cells called peripheral antigen-expressing cells (PAE) in the thymus and can induce central tolerance. While thymic medullary epithelial cells are the prototypic PAE that express peripheral antigens via an *aire*-dependent mechanism, some studies also describe bone marrow (BM)-derived dendritic cells (DC) and macrophages as PAE in both the thymus and secondary lymphoid organs. However, the role of these cells in development of tolerance to tissue-specific antigens has not been elucidated. Here we use BM radiation chimeric mice to study the existence of hematopoietic PAE and their contribution to tolerance to tissue-specific antigens. Our results reveal that BM-derived PAE exist in both central and secondary lymphoid organs and that the expression of peripheral antigens in the BM-derived cells does not correlate with *aire* expression. Using double-transgenic mice expressing TCR specific for a model antigen expressed under the control of a prostate-specific promoter, we show that expression of self antigen in PAE of non-hematopoietic origin is both necessary and sufficient to induce clonal deletion. Surprisingly, while BM-derived PAE fail to induce clonal deletion, they do cause the activation-induced cell death of autoreactive cells in the secondary lymphoid organs. Thus, BM-derived PAE have a distinct function in the maintenance of tolerance to tissue-specific antigens.

| | |
|---|---|
| Received | 2/4/04 |
| Revised | 22/6/04 |
| Accepted | 19/7/04 |

**Key words:** Thymus / T cell development / Peripheral antigen expressing cells / Tolerance

## 1 Introduction

The expression of peripheral antigen in the thymus by PAE serves to project a "shadow of immunological self" in the thymus [1]. By constitutively expressing antigens assumed to be limited in the peripheral organs, PAE ensure that tolerance to tissue-specific antigens is imposed during T cell development and thus reassert the importance of central tolerance in self-nonself discrimination [2–10].

Recent studies established medullar thymic epithelial cells (mTEC) as the prototypic PAE cell phenotype in the thymus [6–9]. The expression of a diverse set of genes that encompass cell surface proteins, enzymes, hormones, and structural proteins, which are all restricted tissue distribution, has been detected in mTEC [9, 11, 12]. Expression of these genes correlates to the risk of autoimmunity in experimental models of autoimmune diseases. More recently, it has been demonstrated that mutation of *aire*, a nuclear protein with preferential expression in mTEC, abrogates the expression of a large array of the peripheral antigens in the thymus [1] and prevents deletion of T cells specific for antigens expressed under the control of tissue-specific promoters [13].

Meanwhile, it has also been reported that dendritic cells (DC) and macrophages are possible PAE candidates. In support of this contention are thymus cell-fractionation studies [3, 4], which show that thymic insulin-expressing cells segregate into a low-density fraction that is enriched with DC and macrophages. In addition, several reports demonstrated the co-localization of insulin and other pancreatic hormones with markers of the DC and macrophage lineages in the murine and human thymus [5, 10]. However, the functions and mechanisms of these putative PAE in self-tolerance have not been systematically analyzed.

[DOI 10.1002/eji.200425177]

**Abbreviations: PAE:** Peripheral antigen-expressing (cells) **mTEC:** Medullar thymic epithelial cells **TRAMP:** Transgenic adenocarcinoma of mouse prostate **Tag:** Large T antigen **AICD:** Activation-induced cell death

 

In analysis of the mechanism of immune tolerance in the transgenic mouse prostate cancer model (TRAMP), we have demonstrated that DC constitute a substantial portion of thymic PAE expressing the SV40 large T antigen (Tag) under the control of a prostate-specific promoter [14]. While the PAE in TRAMP mice caused complete deletion of SV40 Tag-specific T cells, the subset of PAE responsible for the clonal deletion was not identified. Here we used irradiation chimeric mice to demonstrate that radio-resistant PAE are necessary and sufficient to cause clonal deletion of the Tag-specific T cells. Surprisingly, bone marrow (BM)-derived PAE caused activation-induced cell death (AICD) of self-reactive T cells in the spleen. Our results establish novel function and mechanisms of BM-derived PAE in the induction of tolerance of T cells to tissue-specific antigens in the secondary lymphoid organs.

## 2 Results

### 2.1 Radio-resistant PAE are necessary and sufficient to induce clonal deletion in the thymus

TG-B mice express, at high levels, a T cell receptor from a $CD8^+$ cytotoxic T cell clone that recognizes SV40 Tag presented by the MHC class I molecule H-$2K^K$ [15]. We recently demonstrated that the transgenic T cells are deleted in TRAMP/TG-B double-transgenic (H-$2^{bxk}$) mice due to PAE in the thymus [14]. In this study, chimeric mice were produced by transferring BM from TG-$B^+$ mice (with or without the SV40 Tag transgene) to lethally irradiated TG-$B^-$ mice (with or without SV40 Tag) (Table 1). With the exception of the recipients in group I, all groups of chimeric mice synthesized the SV40 Tag in the non-hematopoietic cells of the recipients and/or in BM-derived donor cells. To confirm that both radio-resistant recipient cells and BM-derived donor cells expressed the Tag in the thymus, we used a previously described method [14], based on RT-PCR plus probing of products by Southern blot, to determine expression of Tag in the thymi of the chimeric mice. As shown in Fig. 1, Tag mRNA was detectable in the thymi of chimeric mice from groups II, III and IV, although the amounts detected in group III were significantly lower than those in groups II and IV. This result is consistent with our immunohistochemical analysis of SV40 Tag protein expression in the thymus [14], which revealed that while both DC and non-DC express Tag, most Tag-expressing cells lack CD11c. As expected, no Tag mRNA was detected in group I thymi.

**Table 1.** Total thymocytes reconstituted from different groups of BM chimeras[a)]

| Group | Donor (TRAMP/TG-B) | Recipient (TRAMP/TG-B) | Thymocytes ($\times 10^6$) |
|---|---|---|---|
| I | –/+ | –/– | 53.1±6.3 |
| II | –/+ | +/– | 31.6±2.5*** |
| III | +/+ | –/– | 49.2±3.5 |
| IV | +/+ | +/– | 25.7±2.7*** |

[a)] Data are shown as mean ± SD and are representative of six independent experiments. (***$p<0.001$ compared with groups I and III).

To analyze the functions of two different lineages of PAE in the thymus, we studied the fate of Tag-reactive T cells by flow cytometry. In comparison to group I, the total thymocytes recovered from reconstituted thymi were also clearly reduced in groups II and IV, but not in group III (Table 1). Moreover, thymi from groups I and III had essentially identical subset distributions, while those from groups II and IV were depleted of $CD8^+CD4^-$ T cells (data not shown). Among the T cells that express high levels of transgenic TCRβ, the reduction of the mature antigen-specific T cells was even more pronounced (Fig. 2A, B). Groups I and III had comparable numbers of antigen-specific T cells with similar responsiveness to peptide stimulation (Fig. 2C). However, a more than five-fold reduction in the number of mature $CD8^+$ T cells was observed in groups II and IV. Since the common feature of groups II and IV is their shared origin of radio-resistant PAE, our results demonstrate that expression of Tag in the recipient thymic stromal cells is sufficient to induce

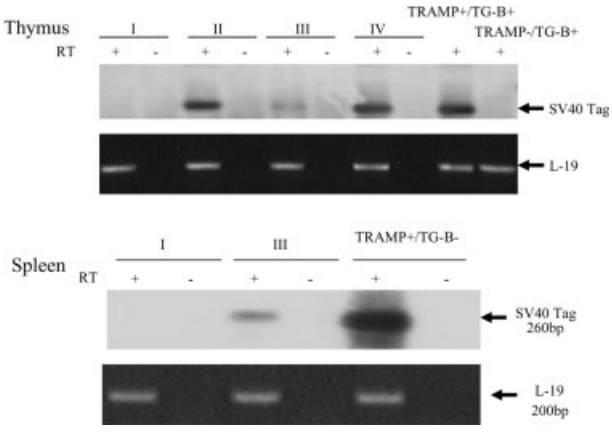


*Fig. 1.* Expression of SV40 Tag mRNA in reconstituted thymus and spleen from recipients of different BM chimeras. RT-PCR results of L-19 amplification (25 cycles) were shown as an agarose gel image. The SV40 Tag PCR products (35 cycles) were separated by agarose gel electrophoresis and transferred to the Hybond N+ membrane. The membrane was hybridized with an HRP-labeled probe, and signals were detected by the ECL direct nucleic acid labeling and detection system (Amersham).

 

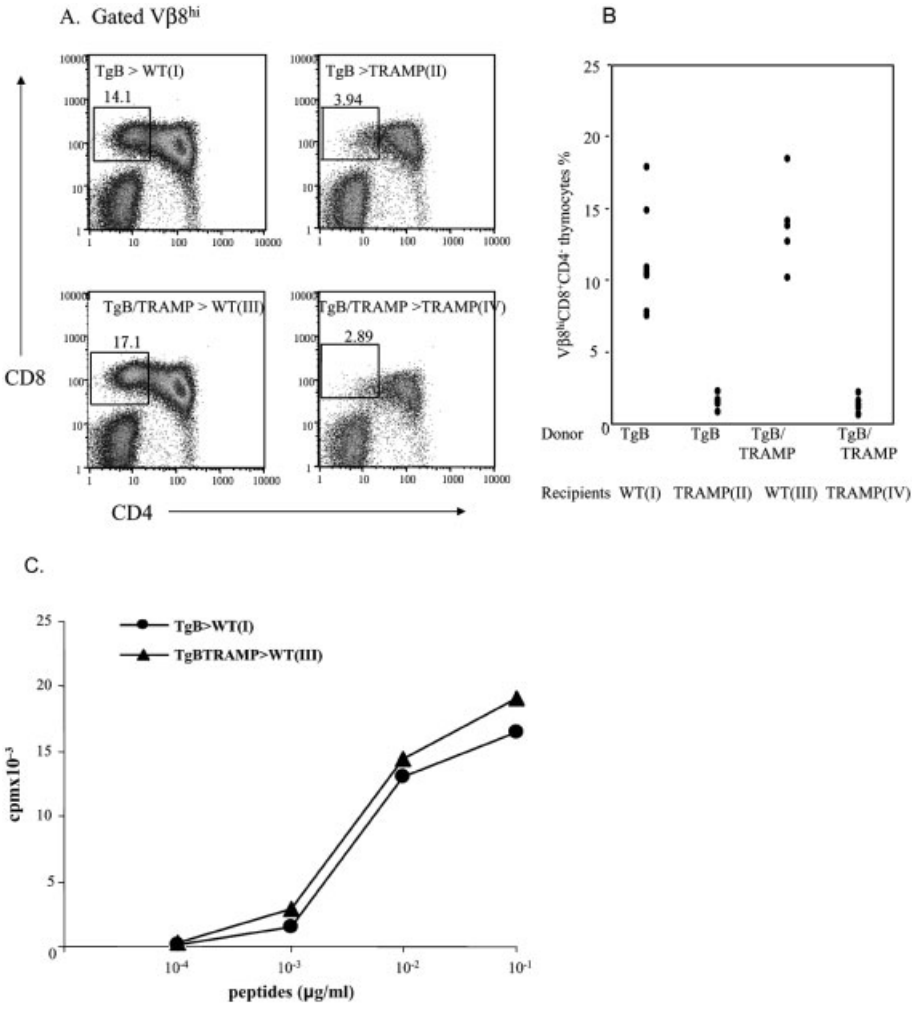


*Fig. 2.* Clonal deletion of Tag-specific T cells in the thymus. Thymi from reconstituted chimeric mice were harvested 8 weeks after BM transplantation. Thymocytes were stained with anti-CD4, anti-CD8, and anti-Vβ8.1/8.2 antibodies. A representative profile of the subset distribution (%) of gated $V\beta8^{high}$ T cells from each group (A) and combined results (B) from six independent experiments (8–12 in each group) are shown ($p<0.001$ when comparing groups II and IV to groups I and III). (C) Thymic T cells from groups I and III respond equally to antigenic peptide *in vitro*. Total thymocytes ($2\times10^5$/well) were harvested from either group III (donor, TG-B/TRAMP; recipient, B6×B10.BR) or group I (donor, TG-B/B6; recipient, B6×B10.BR) chimeric mice and stimulated with SV40 Tag peptides 560–568 in the presence of irradiated B6×B10.BR splenocytes as APC. Results of the proliferation assay from two independent experiments are presented.

clonal deletion. In addition, since no clonal deletion was observed in group III chimeras that have BM-derived PAE, expression of Tag in the recipient radio-resistant PAE is also necessary for the clonal deletion of autoreactive T cells.

## 2.2 BM-derived PAE cause AICD of self-reactive T cells in the secondary lymphoid organs

As shown in Fig. 3, in comparison with group I chimeras, groups II and IV had substantially reduced numbers of transgenic T cells in the spleen. This reduction roughly correlated to that found in the thymi (Fig. 2). Surprisingly, although groups I and III had essentially identical numbers of mature transgenic T cells in the thymi (Fig. 2), the number of transgenic T cells in the spleens of group III chimeras was approximately three- to five-fold lower than found in group I (Fig. 3). These results suggest that BM-derived PAE can reduce the number of autoreactive T cells in the secondary lymphoid organs.

The reduction in the number of T cells resulted in reduced responses of T cells to *in vitro* stimulation by the cognate peptide. As shown in Fig. 4A, spleen cells from groups II and IV did not proliferate in response to the Tag peptide,

 

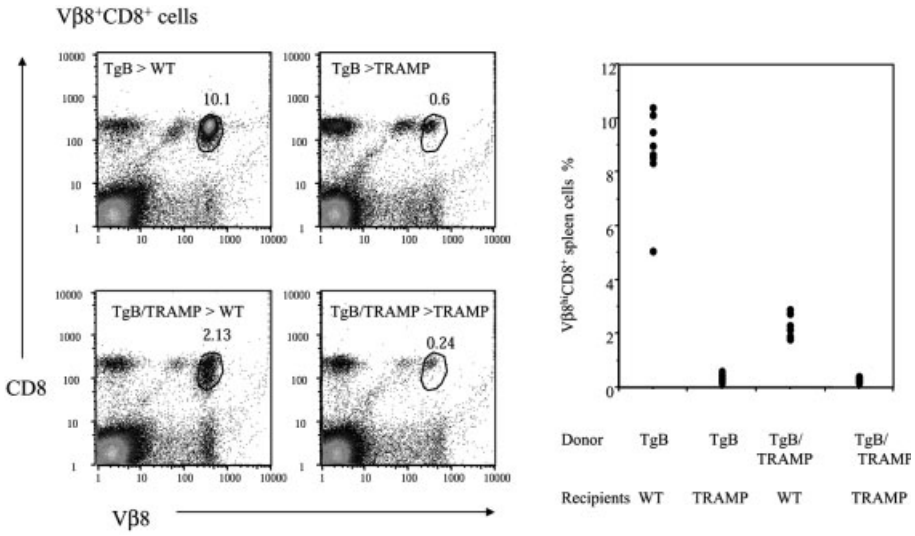


*Fig. 3.* Peripheral deletion of autoreactive T cells by BM-derived PAE in the spleen. The spleens from reconstituted chimeric mice were harvested 8 weeks after BM transplantation. Splenocytes were stained with anti-CD4, anti-CD8, and anti-Vβ8.1/8.2 antibodies. A representative profile the subset distribution (%) of total splenocytes from each group (left) and the combined results (right) from six independent experiments (8–12 in each group) are shown. Note that in some experiments, reconstitution of non-transgenic $CD8^+$ T cells expressing lower levels of Vβ8 can be observed; this population was excluded from the analysis ($p<0.001$ when comparing group I to group III; group II to group I or III; or group IV to groups I or III, respectively; $p<0.01$ when comparing group IV to group II).

while group III mice mounted a significant, although much reduced, proliferation. After *in vitro* stimulation, potent CTL could be elicited from group I, but not group IV, spleen cells (Fig. 4B). In most experiments, a recall CTL response was not detectable in group II spleens, while a much reduced (about 100-fold less as judged by E/T ratio) CTL activity could be elicited from group III. In some experiments, however, a low but significant CTL response was detected in groups II and III. Thus, optimal tolerance to self antigens requires both lineages of PAE.

It has been demonstrated that host APC can cross-present tissue-specific antigens and thereby cause AICD of T cells [16]. However, it has not been tested whether hematopoietic cells can express tissue-specific antigens and induce AICD in the secondary lymphoid organ. To determine if this is the case, we compared spleen cells from groups I and III for activation markers and signs of programmed cell death. As shown in Fig. 5A, the transgenic T cells from group III were clearly being stimulated, as substantial proportions expressed CD69, CD25, and CD24, which are T cell activation markers. Interestingly, the difference in down-regulation of the memory marker CD62L was much less pronounced, which is consistent with the fact that activation of T cells in group III did not lead to stronger CTL recall responses (Fig. 4B).

Instead of inducing strong memory markers, we observed that group III T cells in the spleen had elevated expression of Fas and Fas ligand, which were critical for AICD (Fig. 5B, C). Tunnel assay revealed about a five-fold increase in the proportion of cells undergoing programmed cell death (Fig. 5D). This result demonstrates that PAE of hematopoietic origin induce AICD of self-reactive T cells.

### 2.3 Peripheral antigen expression by PAE in the secondary lymphoid organs does not correlate with *aire* expression

It has been demonstrated that *aire* is preferentially expressed in the mTEC and functions as a transcriptional regulator to control the peripheral organ-specific antigen expression in the thymus [1, 13]. Interestingly, significant expression of *aire* can be detected in the spleen, although the level is about 10% of what is found in the thymus [1]. To determine whether *aire* expression correlates with the synthesis of autoantigens in the secondary lymphoid organs, we compared $CD11c^+$, $CD11c^-$, and total spleen cells for expression of *aire* and a panel of autoantigens that are found in the PAE in the thymus. As shown in Fig. 6, enrichment of $CD11c^+$ cells increased the *aire* mRNA by about ten-fold, while elimination of the $CD11c^+$ cells reduced *aire* mRNA by more than ten-fold. Thus, DC are the primary *aire*-expressing cells in the spleen. SV40 Tag expression was neither enriched nor depleted in the $CD11c^+$ population

 

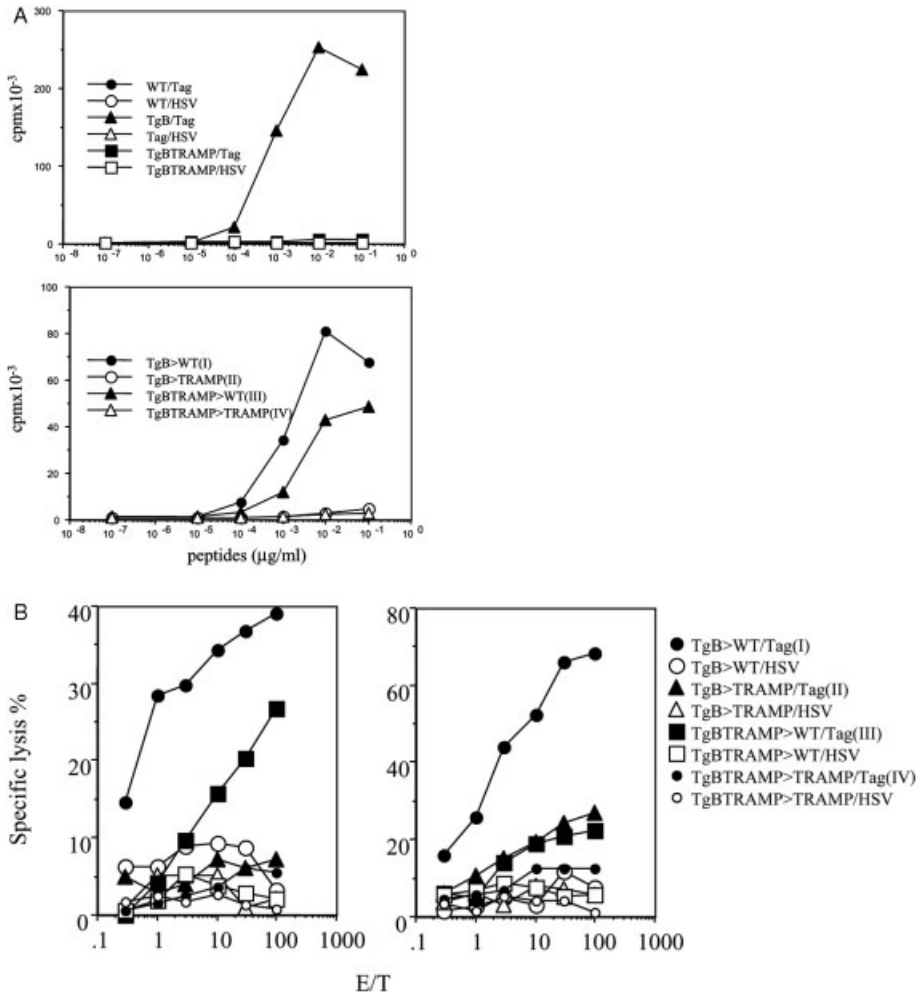


*Fig. 4.* The functions of mature SV40 Tag-specific T cells in the spleen. The spleens from reconstituted chimeric mice were harvested 8 weeks after BM transplantation. Data from assays of proliferation (A) and cytotoxicity (B) in response to SV40 Tag peptide 560–568 and the control peptide HSV-gB from six independent experiments (8–12 in each group) are presented. Representative experiments reflecting two patterns of CTL responses are presented in Fig. 4B.

(Fig. 6A). Of the three "organ-specific" autoantigens that are found in mTEC but not in DC and macrophages in the thymus [17], insulin and cytochrome P450 1a2 mRNA were found at significant and comparable levels in total spleen cells as well as $CD11c^+$ and $CD11c^-$ spleen cells. In contrast, GAD67 mRNA was barely detectable in $CD11c^+$ cells, and depletion of $CD11c^+$ cells did not reduce GAD67 mRNA (Fig. 6B).

We further isolated mRNA from TRAMP mice and compared the RNA expression of *aire,* SV40 Tag, insulin, P450, and an endogenous mouse prostate protein probasin (mPB) in thymus and spleen. As reported before [1, 13], the *aire* expression in thymus was ten-fold more than in the spleen. The insulin and P450 showed higher mRNA expression in the thymus, while the SV40 Tag and endogenous prostate protein mPB showed higher mRNA expression in the spleen (Fig. 6C). These results make two points. First, expression of tissue-specific antigens in the spleen is not limited to the SV40 Tag transgene. Secondly, the expression of "organ-specific" autoantigens does not correlate with *aire* expression in the spleen.

## 3 Discussion

PAE in the thymus constitutively express antigens assumed to be limited to the peripheral organs [4]. Although thymic medullar epithelial cells are considered the major PAE in the thymus [7, 9], several groups have reported *de novo* synthesis of peripheral antigens in hematopoietic cells in the thymus [5, 10, 18]. However, the relative contribution of the two types of PAE for clonal

 

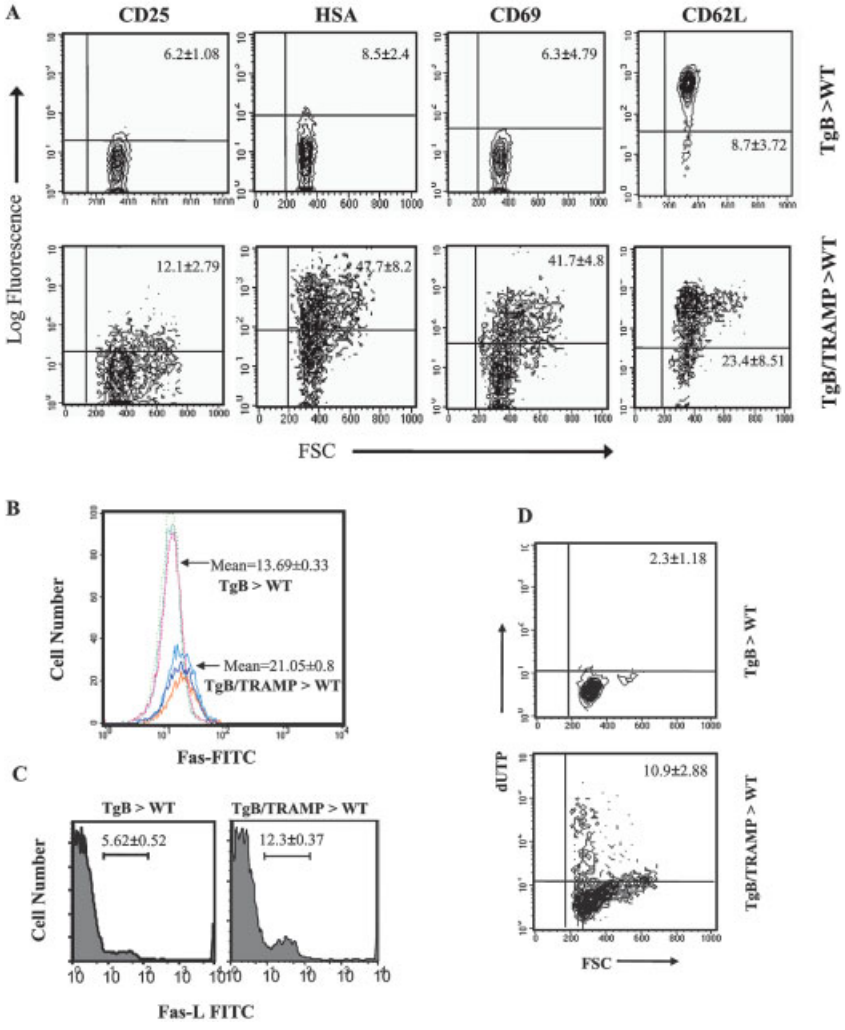


*Fig. 5.* BM-derived PAE cause AICD of self-reactive T cells in the secondary lymphoid organs. The spleens from reconstituted chimeric mice were harvested 8 weeks after BM transplantation. Splenocytes were stained with anti-CD4, anti-CD8, anti-Vβ8.1/8.2, and antibodies against different activation markers (A), Fas (B), or Fas-L (C). Cells undergoing apoptosis were visualized using FITC-conjugated dUTP (D). The numbers in the panels are the percentage of the population (A, C, and D) or mean fluorescence intensity (B) of the gated $CD8^{+}V\beta8^{high}$ T cells.

deletion in the thymus has not been addressed. Our previous study established that TRAMP mice express prostate-specific antigen in both lineages of PAE [14]. In order to identify which subset of PAE induces clonal deletion, we made BM chimeric mice that express the peripheral antigen in only one lineage. Using deletion of SV40 Tag-specific transgenic T cells as a basic readout, we demonstrated that expression of the peripheral antigens in thymic epithelial cells is sufficient to induce clonal deletion. Since no clonal deletion was observed in mice that had only BM-derived PAE, expression of peripheral antigens in the thymic epithelial cells is also necessary for clonal deletion. Given the fact that thymic mTEC are the major PAE in the thymus for the majority of antigens analyzed [9], our conclusion may be generally applicable to clonal deletion of tissue-specific antigens. This is also compatible with recent genetic data showing that mutation of *aire*, which prevents the expression of many tissue-specific antigens in the mTEC [1], inhibits clonal deletion of tissue-specific T cells in the thymus [13].

In light of previous reports that the expression of minor H, allogeneic MHC, or viral superantigen in either thymic epithelial cells or BM-derived cells is sufficient to induce clonal deletion [19–22], it is surprising that expression of peripheral antigens by BM-derived cells in the thymus does not cause clonal deletion. Since our analysis indicates that the level of expression is lower among the hematopoietic APC (this study), and since the number of BM-derived PAE is substantially lower than the number of

 

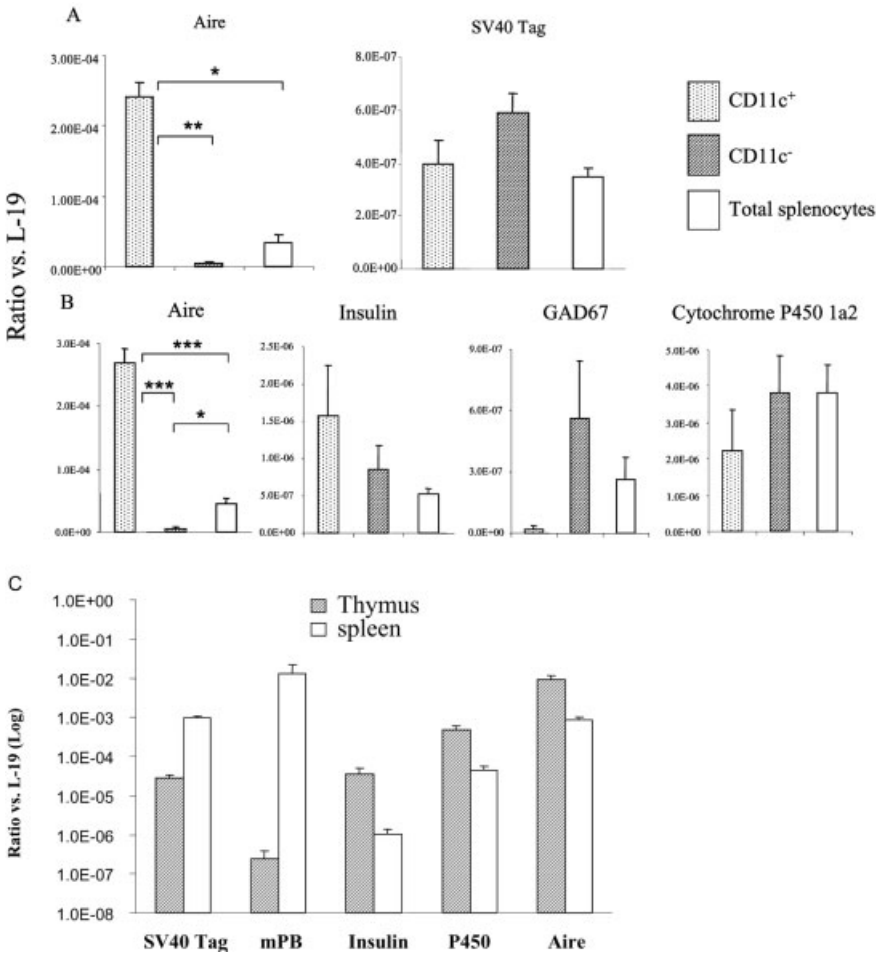


*Fig. 6.* The expression of *aire* does not correlate with that of "organ-specific autoantigens" in the spleen. (A) Expression of *aire* and SV40 Tag in the spleen cells from irradiation chimeras (group III: donor, TG-B/TRAMP; recipient, B6×B10.BR). Data shown are a summary of three independent experiments. (B) Expression of *aire,* insulin, GAD, and cytochrome c P450 in spleens from irradiation chimeras. Data shown are a summary of four independent experiments involving either group III (donor, TG-B/TRAMP; recipient, B6×B10.BR) or group I (donor, TG-B/B6; recipient, B6×B10.BR) chimeric mice. The spleens were harvested and fractionated into $CD11c^+$ and $CD11c^-$ subpopulations. (C) Expression of SV40 Tag, mouse prostate protein probasin (mPB), insulin, cytochrome P450, and *aire* from total thymus or spleen of 9-week-old TRAMP mice. Real-time PCR was carried out to detect the expression levels of different genes. The relative amount of the gene was normalized using the CT values of the sample and the corresponding standard curve. The target gene expression level was quantified using the ratio between the amount of target gene and the housekeeping gene ribosome L-19 within the same sample. Statistical analysis was done with the Student's *t*-test (*$p<0.05$; **$p<0.01$; ***$p<0.001$).

thymic epithelial cells [14], the requirement for *de novo* synthesis by thymic stromal cells may simply be due to their higher expression of peripheral antigen.

Another important observation documented in this study relates to expression and function of PAE in the secondary lymphoid organs. Although the existence of PAE in secondary lymphoid organs had been suggested [10], it was unclear if antigen expression is *aire* dependent. Our data demonstrate that the expression of peripheral antigens by the APC does not correlate with the level of *aire* expression. As such, the expression is unlikely to be controlled by *aire*. Our data further demonstrate that BM-derived PAE reduce the number of autoreactive T cells in periphery and cause AICD of self-reactive T cells in the secondary lymphoid organs. The localization of cells undergoing programmed cell death is consistent with the notion that PAE in the secondary lymphoid organs cause AICD, although an imprinting by thymic hematopoietic PAE during T cell development cannot be ruled out at this stage. These two lines of evidence establish a novel function and mechanism of BM-derived PAE in the induction of tolerance of T cells to tissue-specific antigens. It is likely

 

that this mechanism complements the previously established mechanism by which host APC cross-present tissue antigen to induce AICD [23, 24].

Taken together, we have demonstrated that two populations of PAE play distinct roles in the induction of tolerance of self-reactive T cells. The non-hematopoietic PAE, presumably medullar epithelial cells, induce clonal deletion in the thymus. Perhaps because of lower levels of antigen expression, the hematopoietic PAE are neither necessary nor sufficient to induce clonal deletion in the thymus but induce AICD in the secondary lymphoid organs. Since clonal deletion induced in the thymus is rarely complete under physiological conditions, optimal tolerance may require concerted actions of both populations of PAE.

## 4 Materials and methods

### 4.1 Experimental animals

TRAMP mice expressing the SV40 Tag controlled by rat probasin regulatory elements (C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME). TG-B mice on the B10.BR background were kindly provided by Dr. T. Geiger from St. Jude's Children's Hospital [25]. TRAMP and TG-B mice were bred at the animal facilities of the Ohio State University (Columbus, OH). Mice were typed for SV40 Tag or TCR expression by isolation of mouse tail genomic DNA. The PCR-based screening assays were described previously [14].

### 4.2 Generation of irradiation BM chimeras

Four groups of chimeric mice with different donor and recipient combinations from TRAMP$\times$TG-B (H-$2^{bxk}$) F1 phenotypes are presented in Table 1. Briefly, the lethally irradiated (1,000 rad) mice were reconstituted with BM from femurs and tibias of the donor mice after the T cells were depleted with anti-CD4 (Gk1.5) and anti-CD8 (TIB210) monoclonal antibodies. A total of $1.0\times10^7$ T cell-depleted BM cells were injected i.v. through tail vein into the recipient mouse. All experiments were performed 8 weeks after BM reconstitution.

### 4.3 Antibodies

The fluorescence-conjugated antibodies anti-CD4 (RM4.5), anti-CD8 (53–6.7), anti-V$\beta$8.1/8.2 (MR5–2), anti-CD25 (PC61), anti-HSA (M1/69), anti-CD69 (H1.2F3), anti-CD62L, anti-CD28 (37.51), anti-Fas Ligand (MFL3), anti-Fas (Jo2), and the APO-DIRECT Kit were purchased from BD PharMingen (San Diego, CA).

### 4.4 Peptide synthesis

Peptides (SV40 Tag 560–568 SEFLLEKRI [14] and HSV gB peptide gB498–505 SSIEFARL [26]) were synthesized by Research Genetics, Inc. (Huntsville, AL). The peptides were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml and diluted in PBS or culture medium before use.

### 4.5 Proliferation of T cells to antigenic peptides and CTL assays

The T cell proliferation and CTL assays have been previously described [14].

### 4.6 Fractionation of splenic CD11c$^+$ and CD11c$^-$ cells and real-time PCR

Splenic CD11c$^+$ and CD11c$^-$ cells were fractionated according to a previously described protocol [27]. Briefly, collagenase (Sigma) solution (1 mg/ml in 10 mM Hepes-NaOH, pH 7.4) was injected into the spleen before the spleen was sliced and incubated with additional collagenase solution for 60 min at 37°C. A single-cell suspension was obtained by passing the digested spleen through a steel mesh. Red blood cells were lysed with 1$\times$ lysis buffer (0.15 M $NH_4Cl$, 1.0 mM $KHCO_3$, 0.1 mM $Na_2EDTA$, pH 7.4). The viable cells were used as the total spleen cell population. CD11c$^+$ and CD11c$^-$ cells were magnetically separated using CD11c microbeads and LS$^+$ positive selection columns according to manufacturer's protocol (Miltenyi Biotec Inc., Auburn, CA). Total RNA was extracted from total spleen, CD11c$^+$, and CD11c$^-$ cells, and 1 $\mu$g/sample was pretreated with RNase-free DNase I before cDNA synthesis using Superscriptase II and oligo(dT) (Invitrogen, Carlsbad, CA). The real-time PCR was carried out in ABI PRISM 7700 Cycler (Applied Biosystems, Foster City, CA) using the QuantiTect SYBR green PCR kit (Qiagen) according to manufacturers' protocols. The oligonucleotide primers used in real-time PCR were: SV40 Tag, F: 5′-GCTA-CACTGTTTGTTGCCCA-3′ and R: 5′-CCCCCACATAATT-CAAGCAA-3′; Aire, F: 5′-ACCATGGCAGCTTCTGTCCAG-3′ and R: 5′-GCAGCAGGAGCATCTCCAGAG-3′ [9]; Insulin I, F: 5′-TATAAAGCTGGTGGGCATCC-3′ and R: 5′-GGGACCA-CAAAGATGCTGTT-3′; Insulin II, F: 5′-TTTGTCAAGCAG-CACCTTTG-3′ and R: 5′-GTCTGAAGGTCACCTGCTCC-3′; GAD67, F: 5′-ATCGTGCAAGCAAGGAAGCA-3′ and R: 5′-GCAAGAGACCTCGGATAGAAGAGT-3′; Cytochrome P450 1a2, F: 5′-GCTGCCATATCTAGAGGCCTTCAT-3′ and R: 5′-TGGTTGACCTGCCACTGGTTTA-3′; the ribosome L-19: F: 5′-CTGAAGGTCAAAGGGAATGTG-3′and R: 5′-GGACA-GAGTCTTGTGATCTC-3′. The relative amount of the gene was normalized using the CT values of the sample and the corresponding standard curve. The target gene expression level was quantified using the ratio between the target gene and the ribosome L-19 as a reference gene.

 

**Acknowledgements:** This work was supported by grants from the Department of Defense (DAMD17–03-1-0013 to P. Z.), National Institute of Health (CA82355 to P. Z., CA69091 and CA58033 to Y. L.), and by the Ohio State University Comprehensive Cancer Center. We thank Dr. Yongjun Liu for helpful discussion and Ms. Lynde Shaw for editorial assistance.

## References

1 **Anderson, M. S., Venanzi, E. S., Klein, L., Chen, Z., Berzins, S. P., Turley, S. J., von Boehmer, H., Bronson, R., Dierich, A., Benoist, C. and Mathis, D.,** Projection of an immunological self shadow within the thymus by the aire protein. *Science* 2002. **298:** 1395–1401.

2 **Jolicoeur, C., Hanahan, D. and Smith, K. M.,** T-cell tolerance toward a transgenic beta-cell antigen and transcription of endogenous pancreatic genes in thymus. *Proc. Natl. Acad. Sci. USA* 1994. **91:** 6707–6711.

3 **Smith, K. M., Olson, D. C., Hirose, R. and Hanahan, D.,** Pancreatic gene expression in rare cells of thymic medulla: evidence for functional contribution to T cell tolerance. *Int. Immunol.* 1997. **9:** 1355–1365.

4 **Hanahan, D.,** Peripheral-antigen-expressing cells in thymic medulla: factors in self- tolerance and autoimmunity. *Curr. Opin. Immunol.* 1998. **10:** 656–662.

5 **Throsby, M., Homo-Delarche, F., Chevenne, D., Goya, R., Dardenne, M. and Pleau, J. M.,** Pancreatic hormone expression in the murine thymus: localization in dendritic cells and macrophages. *Endocrinology* 1998. **139:** 2399–2406.

6 **Klein, L., Klugmann, M., Nave, K. A., Tuohy, V. K. and Kyewski, B.** Shaping of the autoreactive T-cell repertoire by a splice variant of self protein expressed in thymic epithelial cells. *Nat. Med.* 2000. **6:** 56–61.

7 **Klein, L. and Kyewski, B.,** "Promiscuous" expression of tissue antigens in the thymus: a key to T- cell tolerance and autoimmunity? *J. Mol. Med.* 2000. **78:** 483–494.

8 **Klein, L., Roettinger, B. and Kyewski, B.,** Sampling of complementing self-antigen pools by thymic stromal cells maximizes the scope of central T cell tolerance. *Eur. J. Immunol.* 2001. **31:** 2476–2486.

9 **Derbinski, J., Schulte, A., Kyewski, B. and Klein, L.,** Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat. Immunol.* 2001. **2:** 1032–1039.

10 **Pugliese, A., Brown, D., Garza, D., Murchison, D., Zeller, M., Redondo, M., Diez, J., Eisenbarth, G. S., Patel, D. D. and Ricordi, C.,** Self-antigen-presenting cells expressing diabetes-associated autoantigens exist in both thymus and peripheral lymphoid organs. *J. Clin. Invest.* 2001. **107:** 555–564.

11 **Heath, V. L., Moore, N. C., Parnell, S. M. and Mason, D. W.,** Intrathymic expression of genes involved in organ specific autoimmune disease. *J. Autoimmun.* 1998. **11:** 309–318.

12 **Oukka, M., Colucci-Guyon, E., Tran, P. L., Cohen-Tannoudji, M., Babinet, C., Lotteau, V. and Kosmatopoulos, K.,** CD4 T cell tolerance to nuclear proteins induced by medullary thymic epithelium. *Immunity* 1996. **4:** 545–553.

13 **Liston, A., Lesage, S., Wilson, J., Peltonen, L. and Goodnow, C. C.,** Aire regulates negative selection of organ-specific T cells. *Nat. Immunol.* 2003. **4:** 350–354.

14 **Zheng, X., Gao, J. X., Zhang, H., Geiger, T. L., Liu, Y. and Zheng, P.,** Clonal deletion of simian virus 40 large T antigen-specific T cells in the transgenic adenocarcinoma of mouse prostate mice: an important role for clonal deletion in shaping the repertoire of T cells specific for antigens overexpressed in solid tumors. *J. Immunol.* 2002. **169:** 4761–4769.

15 **Molldrem, J. J., Lee, P. P., Wang, C., Felio, K., Kantarjian, H. M., Champlin, R. E. and Davis, M. M.,** Evidence that specific T lymphocytes may participate in the elimination of chronic myelogenous leukemia. *Nat. Med.* 2000. **6:** 1018–1023.

16 **Kurts, C., Kosaka, H., Carbone, F. R., Miller, J. F. and Heath, W. R.,** Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive CD8(+) T cells. *J. Exp. Med.* 1997. **186:** 239–245.

17 **Kyewski, B., Derbinski, J., Gotter, J. and Klein, L.,** Promiscuous gene expression and central T-cell tolerance: more than meets the eye. *Trends Immunol.* 2002. **23:** 364–371.

18 **Pugliese, A.,** Peripheral antigen-expressing cells and autoimmunity. *Endocrinol. Metab. Clin. North. Am.* 2002. **31:** 411–430, viii.

19 **Lo, D. and Sprent, J.,** Identity of cells that imprint H-2-restricted T-cell specificity in the thymus. *Nature* 1986. **319:** 672–675.

20 **von Boehmer, H., Crisanti, A., Kisielow, P. and Haas, W.,** Absence of growth by most receptor-expressing fetal thymocytes in the presence of interleukin-2. *Nature* 1985. **314:** 539–540.

21 **Gao, E. K., Lo, D. and Sprent, J.,** Strong T cell tolerance in parent→F1 bone marrow chimeras prepared with supralethal irradiation. Evidence for clonal deletion and anergy. *J. Exp. Med.* 1990. **171:** 1101–1121.

22 **Webb, S. R. and Sprent, J.,** Tolerogenicity of thymic epithelium. *Eur. J. Immunol.* 1990. **20:** 2525–2528.

23 **Davey, G. M., Kurts, C., Miller, J. F., Bouillet, P., Strasser, A., Brooks, A. G., Carbone, F. R. and Heath, W. R.,** Peripheral deletion of autoreactive CD8 T cells by cross presentation of self-antigen occurs by a Bcl-2-inhibitable pathway mediated by Bim. *J. Exp. Med.* 2002. **196:** 947–955.

24 **Adler, A. J., Marsh, D. W., Yochum, G. S., Guzzo, J. L., Nigam, A., Nelson, W. G. and Pardoll, D. M.,** CD4+ T cell tolerance to parenchymal self-antigens requires presentation by bone marrow-derived antigen-presenting cells. *J. Exp. Med.* 1998. **187:** 1555–1564.

25 **Geiger, T., Gooding, L. R. and Flavell, R. A.,** T-cell responsiveness to an oncogenic peripheral protein and spontaneous autoimmunity in transgenic mice. *Proc. Natl. Acad. Sci. USA* 1992. **89:** 2985–2989.

26 **Bonneau, R. H., Fu, T. M. and Tevethia, S. S.,** *In vivo* priming and activation of memory cytotoxic T-lymphocytes (CTL) by a chimeric simian virus 40 T antigen expressing an eight amino acid residue herpes simplex virus gB CTL epitope. *Virology* 1993. **197:** 782–787.

27 **Nonacs, R., Humborg, C., Tam, J. P. and Steinman, R. M.,** Mechanisms of mouse spleen dendritic cell function in the generation of influenza-specific, cytolytic T lymphocytes. *J. Exp. Med.* 1992. **176:** 519–529.

**Correspondence:** Pan Zheng, Division of Cancer Immunology, Department of Pathology and Comprehensive Cancer Center, The Ohio State University Medical Center, 129 Hamilton Hall, 1645 Neil Avenue, Columbus, OH 43210, USA
Fax: +1-614-688-8152
e-mail: zheng-1@medctr.osu.edu

 

# B7-CD28 Interaction Promotes Proliferation and Survival but Suppresses Differentiation of $CD4^-CD8^-$ T Cells in the Thymus[1]

**Xincheng Zheng,[2]* Jian-Xin Gao,[2]* Xing Chang,* Yin Wang,* Yan Liu,* Jing Wen,* Huiming Zhang,* Jian Zhang,† Yang Liu,[3]* and Pan Zheng***

**Costimulatory molecules play critical roles in the induction and effector function of T cells. More recent studies reveal that costimulatory molecules enhance clonal deletion of autoreactive T cells as well as generation and homeostasis of the $CD25^+CD4^+$ regulatory T cells. However, it is unclear whether the costimulatory molecules play any role in the proliferation and differentiation of T cells before they acquire MHC-restricted TCR. In this study, we report that targeted mutations of B7-1 and B7-2 substantially reduce the proliferation and survival of $CD4^-CD8^-$ (double-negative (DN)) T cells in the thymus. Perhaps as a result of reduced proliferation, the accumulation of RAG-2 protein in the DN thymocytes is increased in B7-deficient mice, which may explain the increased expression of TCR gene and accelerated transition of $CD25^+CD44^-$ (DN3) to $CD25^-CD44^-$ (DN4) stage. Qualitatively similar, but quantitatively less striking effects were observed in mice with a targeted mutation of CD28, but not CTLA4. Taken together, our results demonstrate that the development of DN in the thymus is subject to modulation by the B7-CD28 costimulatory pathway.** ***The Journal of Immunology*****, 2004, 173: 2253–2261.**

T cells are educated in the thymus to gain immune competence. Mature T cells migrate into secondary lymphoid organs where they encounter Ags, expand, and differentiate into effector cells. The activated T cells are dispatched to target tissues to mediate effector function. As the major costimulators in T cell activation, B7-1 and B7-2 were first demonstrated to play a major role in the activation and differentiation of T cells in the secondary lymphoid tissues (1–3). Subsequently, the notion of T cell costimulation has been extended to T cell effector function in target tissues, including tumors (4–7) and normal tissues during autoimmune destruction (8, 9). Accumulating data from several groups, including that of ours (10), have shown that B7/CD28 enhances clonal deletion of autoreactive T cells (11–14). In addition, anti-CD28 Ab promotes differentiation of $CD4^+CD8^+$ double-positive (DP)[4] thymocytes into single-positive (SP) T cells (15, 16). Moreover, it has been shown that costimulation is critical for the generation and homeostasis of the $CD25^+CD4^+$ regulatory T cells (17–20). Because all of these stages involve interaction of Ag-specific receptors with MHC:peptide complex, it can be suggested that T cell costimulation is an important parameter whenever TCR is engaged by MHC:peptide complex. An interesting issue that has not been addressed is whether T cell costimulation participates in the $\beta$-selection, in which the rearranged TCR$\beta$ are paired with pT$\alpha$ to mediate proliferation and differentiation of immature thymocytes.

The earliest T cell progenitor expresses neither TCR nor coreceptor CD4 or CD8, and is usually referred to as double-negative (DN) T cells for the lack of CD4 and CD8. They are subdivided according to the expression of surface markers CD44 and CD25. DN1, which is $CD44^+CD25^-$, contains cells that are committed to lymphoid lineage, but maintains the potential to develop into T cells, B cells, or NK cells (21). With the increased CD25 expression, DN1 becomes $CD44^+CD25^+$, which are called DN2 cells. Cells at this stage are committed to T cell lineage, and therefore are also called pro-T cells. Growth factors, such as IL-7 and stem cell factor (c-*kit* ligand), play important roles in this developmental step (22–26). DN3, or early pre-T population, down-regulates CD44 and is characterized by $CD44^-CD25^+$. At this stage, the TCR$\beta$ locus is rearranged by a *RAG*-dependent mechanism. This leads to the assembly of the pre-TCR complex consisting of CD3, pT$\alpha$, and TCR$\beta$ chains. Disruption of the complex causes a complete arrest at DN3, as shown in *RAG* (27–29)-, TCR$\beta$ (30)-, and pT$\alpha$ (31)-deficient mice. As further maturation occurs, cells lose expression of CD25 to become $CD44^-CD25^-$ or DN4. DN4 progresses to the $CD4^+CD8^+$ DP via an immature SP stage and then goes through positive and negative selection to become $CD4^+$ or $CD8^+$ SP T cells.

Although it is clear that the survival of DN4 requires rearrangement of TCR$\beta$ and expression of pT$\alpha$, very little is known about other cell surface interactions during the early phase of T cell maturation, which is generally coupled with rapid T cell proliferation. In the process of studying the effect of B7 blockade on the development of Ag-specific T cells in the thymus, we observed that anti-B7 Abs have significant effect on the development of early T cell progenitors. To substantiate this observation, we systematically analyzed the maturation and proliferation of the T cell progenitors in mice with targeted mutation of B7-1 and B7-2,

*Division of Cancer Immunology, Department of Pathology, Ohio State University Medical Center and Comprehensive Cancer Center, Columbus, OH 43210; and †Department of Orthopedic Surgery, Rush University Medical Center, Chicago, IL 60612

Received for publication March 1, 2004. Accepted for publication June 1, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work is supported by National Institutes of Health Grants AI51342 and CA58033 (to Y.L.), a grant from Department of Defense Prostate Cancer Program (DAMD 17-03-1-0013 to P.Z.), and National Institutes of Health Grants P01 AR045652 and K02 AR049047 (to J.Z.).

[2] X.Z. and J.-X.G. contributed equally to this study.

[3] Address correspondence and reprint requests to Dr. Yang Liu, Division of Cancer Immunology, Department of Pathology, Ohio State University Medical Center, 129 Hamilton Hall, 1645 Neil Avenue, Columbus, OH 43210. E-mail address: liu-3@medctr.osu.edu

[4] Abbreviations used in this paper: DP, double positive; $C_T$, threshold cycle; DN, double negative; KO, knockout; SP, single positive; WT, wild type.



0022-1767/04/$02.00

CD28, and CTLA-4. Our results demonstrate targeted mutations of B7-1 and B7-2 or CD28 diminish the proliferation and survival of DN4 T cells and accelerate DN3 to DN4 transition, most likely by increasing accumulation of the RAG-2 protein and enhancing TCR rearrangement.

## Materials and Methods

### *Experimental animals*

Wild-type (WT), B7-1$^{-/-}$B7-2$^{-/-}$ (32), CD28$^{-/-}$ (33) C57BL6/j mice were purchased from The Jackson Laboratory (Bar Harbor, ME). CTLA-4$^{+/-}$ mice in B6 background have been described (34). All animals were maintained in the University Laboratory Animal Research Facility at Ohio State University under specific pathogen-free conditions.

### *Abs and flow cytometry*

Both cell surface markers and intracellular staining were analyzed by flow cytometry. The fluorescence-conjugated Abs anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-CD44 (IM7), anti-CD25 (PC61), anti-CD28 (37.57), anti-CTLA4 (UC10-4F10-11), and anti-TCR β-chain (H57-597) were purchased from BD Pharmingen (San Diego, CA). The fixation and permeabilization solution kit (cytofix/cytoperm; BD Pharmingen) was used for intracellular staining, according to manufacturer's protocol.

To measure the proliferation of thymocytes in vivo, mice were injected i.p. with BrdU (1 mg/mouse in 100 μl of PBS). Four hours later, the mice were sacrificed and thymocytes were prepared. BrdU incorporation was detected by flow cytometry with a BrdU Flow Kit, as described by manufacturer (BD Pharmingen).

The apoptotic thymocytes were determined by their binding to annexin V. After cell surface staining, the cells were resuspended in staining buffer with annexin V (BD Pharmingen) and were stained at room temperature for 15 min. The samples were analyzed by flow cytometry within 1 h.

### *Western blot and real-time RT-PCR*

The total thymocytes were depleted twice with anti-CD4 (GK1.5), anti-CD8 (2.4.3), anti-TCRγδ (GL-3), anti-I-A$^b$ (AF6-120.1), and magnetic Dynabeads (Dynal Biotech, Oslo, Norway), according to manufacturer's manual. The RAG-2 protein level was detected by Western blot. Briefly, cells were lysed in a lysis buffer consisting of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM DTT,1 mM $Na_3O_4$, 2 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF. Total protein extracts (100 μg) were fractionated on a 12% SDS-PAGE and transferred to Hybond-P membrane (Amersham Biosciences, Buckinghamshire, U.K.). The transferred membrane was blotted by a goat polyclonal Ab against RAG-2 (Santa Cruz Biotechnology, Santa Cruz, CA). Mouse β-actin mAb (Sigma-Aldrich, St. Louis, MO) was used for internal blotting control.

Total RNA was extracted from DN thymocytes and treated with RNase-free DNase I (Invitrogen Life Technologies, Carlsbad, CA). cDNA was synthesized with Superscriptase II and oligo(dT) (Invitrogen Life Technologies). The real-time PCR was conducted in ABI PRISM 7700 Cycler (Applied Biosystems, Foster City, CA) using a QuantiTect SYBR green PCR kit (Qiagen, Valencia, CA), according to manufacturers' protocols and real-time PCR conditions. The relative expression levels in B7 knockout (B7KO) thymocytes were compared with those from WT thymocytes after normalization with internal control (ribosome L-19), as follows: comparative expression level $= 2^{-(\Delta C_T B7KO - \Delta C_T B6)}$. $\Delta C_T = C_T$(target gene) $- C_T$(L-19) and represents the difference between the two threshold cycle ($C_T$) values of two PCRs for the same initial template amount. The oligonucleotide primers were: the RAG-1 forward primer, 5′-TGCAGAC ATTCTAGCACTCTGG-3′, and reverse primer, 5′-ACATCTGCCT TCACGTCGAT-3′; the RAG-2 forward primer, 5′-CACATCCACAAG CAGGAAGTACAC-3′, and reverse primer, 5′-TCCCTCGACTATACACCA CGTCAA-3′; the pre-TCRα forward primer, 5′-AGCTTCTGGCTGCA ACTGGGTCAT-3′, and reverse primer, 5′-TACCTGCCGCTGTGTCC CCCCGAG; TCR Vα2 forward primer, 5′-CAATAAAAGGGA GAAAAAGC-3′, and reverse primer, 5′-AAGTCGGTGAACAGGCAGAG 3′; TCR Vα4 forward primer, 5′-AGCAGCAGAGGKTTTGAAGC-3′, and reverse primer, 5′-GGCACATTGATTTGGGAGTC-3′ (35); CD8 β-chain forward primer, 5′-CTGCTTTGAACTGCTGCAAG-3′, and reverse primer, 5′-GGAAGAGTACATGGTGCGT-3′; the ribosome L-19 forward primer, 5′-CTGAAGGTCAAAGGGAATGTG-3′, and reverse primer, 5′-GGACAGAGTCTTGTGATCTC-3′ (36), was used as internal control.

### *Antimitotic drug treatment*

Eight-week-old C57BL/6 mice were given three injections of either demecolcine (Sigma-Aldrich) at 200 μg/mouse/injection or PBS at 4-h intervals. This was followed by two daily treatments with the same amount of drug. The BrdU was injected in conjunction with the last treatment. The mice were sacrificed at 4 h after the last injection to harvest thymocytes for analyses.

### *Immunohistochemistry with anti-B7-1 or anti-B7-2 mAb*

Frozen sections of the thymus fixed with acetone and were incubated with anti-B7-1 mAb 3A12 (37) or anti-B7-2 mAb GL-1 (38) hybridoma supernatants. The anti-B7-1 mAb was detected by biotinylated goat anti-hamster Abs, while anti-B7-2 Abs were detected by biotinylated goat anti-rat Abs, each followed by HRP-conjugated streptavidin.

### *Statistical analysis*

Data were statistically analyzed with two-tailed Student's *t* test.

## Results

### *Impact of defective B7-1, B7-2, and their receptors on the DN subsets*

In the process of studying the effect of anti-B7-1/2 Abs for T cell development in vivo, we observed that anti-B7-1/2, but not control Ig, caused a substantial reduction of DN3 and a major increase of DN4 in thymus of adult C57BL/6 mice and perinatal BALB/c mice. A statistically significant reduction was also observed in the DN2 subset (data not shown). To substantiate this observation, we compared mice deficient for B7-1 and B7-2 with their WT controls for DN subsets. As shown in Fig. 1, in comparison with WT, B7-deficient mice had an increased DN4, but decreased DN2 and DN3.

B7-1 and B7-2 interact with two known receptors, CD28 and CTLA-4, on T cells. A putative third receptor has been suggested (39, 40), although its identity remains elusive. As a first step to determining the receptors that may be involved in the alteration of DN3 and DN4, we first analyzed cell surface expression of CD28 and intracellular accumulation of the CTLA-4 among DN subsets of WT thymus. As shown in Fig. 2, in comparison with isotype control, CD28 is expressed on ~13% of DN3 and 70–80% of DN4. CTLA-4, in contrast, is expressed on ~80% of DN3 cells and 40% of DN4. To determine the role for CD28 and CTLA-4 in the development of DN, we compared WT, B7KO, CD28KO, and CTLA-4KO for the DN subsets. As shown in Fig. 1, targeted mutation of CD28 had significant effects on the distribution of DN. Qualitatively, reductions in DN2 and DN3 and an increase in DN4 were parallel to what was found in B7$^{-/-}$. However, targeted mutation of CD28 was less effective than those of the B7-1 and B7-2. To determine whether CTLA4 function explains the differences between CD28$^{-/-}$ and B7$^{-/-}$, we compared thymi harvested from 15-day-old CTLA-4$^{+/-}$ and CTLA-4$^{-/-}$ mice for DN maturation and proliferation. At this point, the overall subset distribution of SP, DN, and DP subsets is grossly normal (41) and no autoimmune disease was observed. Fig. 3 shows the profiles of DN thymocytes in 2-wk-old CD28$^{-/-}$, B7$^{-/-}$, CTLA-4$^{+/-}$, and CTLA-4$^{-/-}$ mice. The distribution of DN subsets in the 2-wk-old CTLA4$^{+/-}$ mice was similar to that of adult mice (Fig. 1). No statistically significant difference was detected in the relative amounts of DN subsets between CTLA4$^{+/-}$ and CTLA4$^{-/-}$ mice. Mutation of B7 and CD28, in contrast, significantly reduced DN3 and increased DN4. In contrast to what was observed in adult mice (Fig. 1), the effects of B7 mutation in the young mice were comparable to those of CD28. Thus, CTLA-4 alone is not responsible for the costimulation by B7-1 and B7-2 in the early stage of thymocyte development.

### *Role for B7-1/2 and CD28 in proliferation and programmed cell death of DN*

An important feature of the DN thymocytes is their high rate of proliferation (42, 43). To test whether B7 deficiency affects its

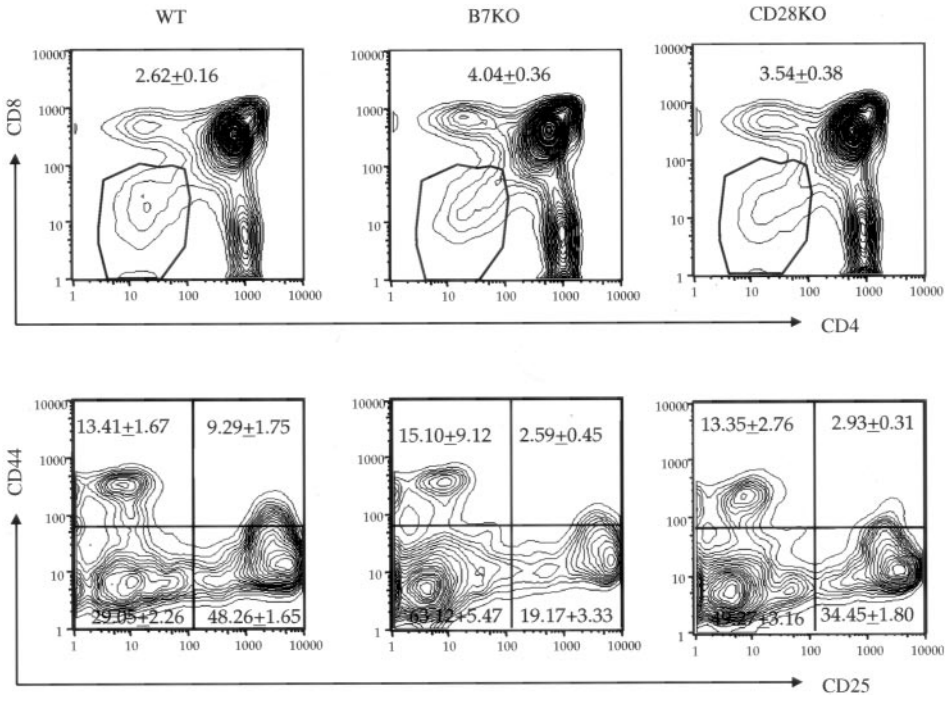


**FIGURE 1.** The distribution of DN subsets in the thymi of WT, B7KO, and CD28KO mice. B7KO, CD28KO, and C57BL/6 mice were sacrificed at 8 wk old, and thymocytes were harvested and stained with anti-CD4, anti-CD8, anti-CD44, and anti-CD25 Abs. Profiles of total viable cells (*upper panels*) or gated $CD4^{-}CD8^{-}$ DN populations (*lower panels*) are shown. Contour graphs depict thymocyte subsets of one mouse in each group. Means and SD are given in the panels. The experiments have been done twice, using a total of nine mice per group.

proliferation, we pulsed WT, CD28-, and B7-deficient mice with BrdU and measured the DNA synthesis of the ex vivo DN by flow cytometry 4 h later. As shown in Fig. 4, in agreement with previous publications (44, 45), two major waves of proliferation were observed in DN2 (~20%) and DN4 (~40%) in the WT mice, and somewhat less pronounced, but still substantial proliferation was found in DN3 (~10%). Interestingly, <8% of the DN4 from B7-deficient mice incorporated BrdU. As expected, this reduction corresponds to a reduction of the proportion of DN4 with large forward scatters. Thus, targeted mutations of B7-1 and B7-2 suppress both enlargement and DNA synthesis of the DN4 cells. Although less pronounced than what was found in the B7-deficient mice, DN4 from CD28-deficient mice also showed a ~2-fold reduction in the BrdU incorporation.

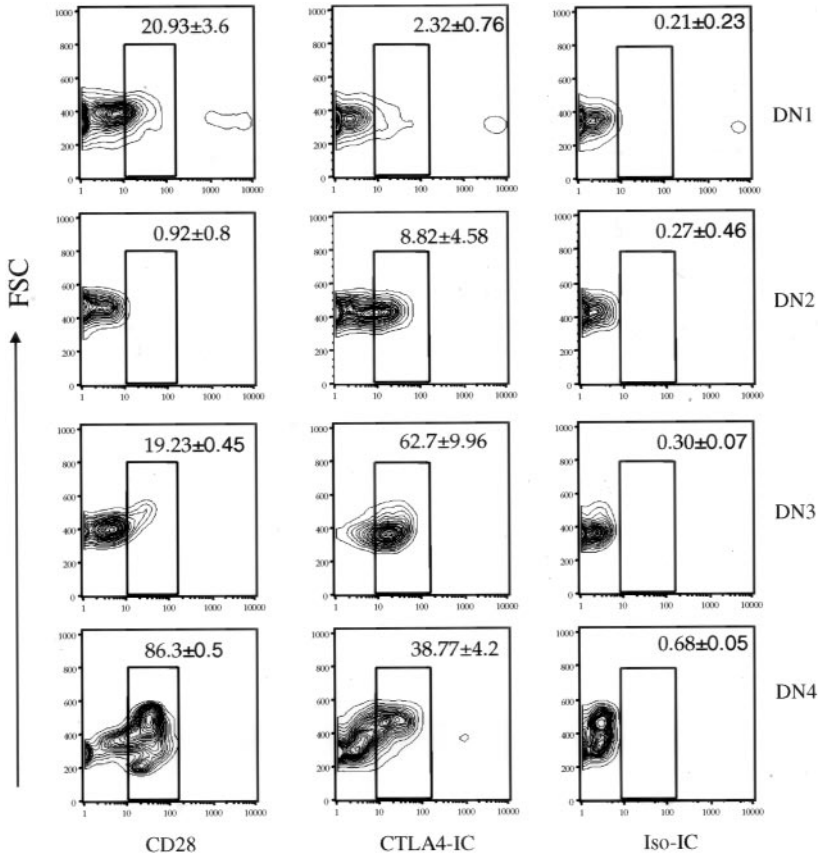


**FIGURE 2.** The expression of CD28 and CTLA-4 on different DN subsets. WT C57BL/6 mice were sacrificed at 8 wk old, and thymocytes were harvested. Cells were surface stained with anti-CD4/CD8, anti-CD44, anti-CD25, and anti-CD28, and intracellularly stained with anti-CTLA-4 Abs. The DN were identified as the negative group in a staining using a mixture of anti-CD4 and anti-CD8 mAbs with the same fluorochrome. The number indicates the percentage of cells that bind Abs specific for CD28 and CTLA-4 or isotype control Ig (hamster IgG1). Data shown are contour graphs of one representative mouse from each group of three mice, and the numbers in the squares are the means and SD of the percentage of the subsets. This experiment has been repeated twice.

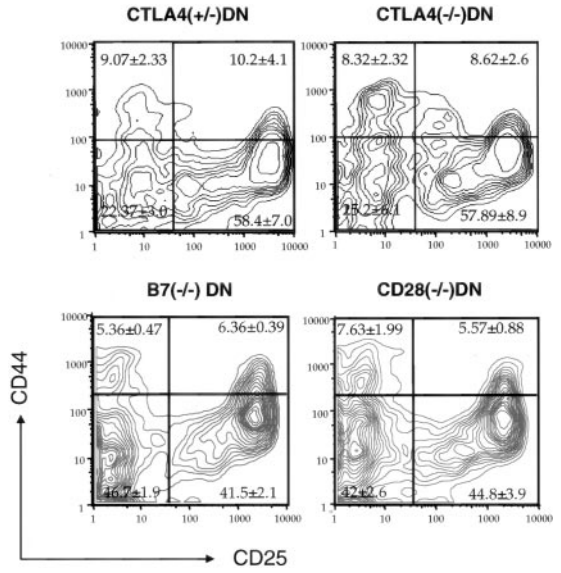


**FIGURE 3.** CTLA-4 alone is not responsible for the effect of B7-1/2 in DN thymocyte development. Thymi from 2-wk-old CTLA-$4^{+/-}$ ($n = 8$), CTLA-$4^{-/-}$ ($n = 7$), $B7^{-/-}$ ($n = 9$), and $CD28^{-/-}$ ($n = 8$) mice were harvested and stained with anti-CD4, anti-CD8, anti-CD44, and anti-CD25 Abs. Percentages shown in the quadrants are summaries of data from two independent experiments. No significant difference in DN subsets was observed between CTLA$4^{+/-}$ and CTLA$4^{-/-}$ mice. The differences between $B7^{-/-}$ and CTLA$4^{+/-}$ or $CD28^{-/-}$ and CTLA$4^{+/-}$ are highly significant ($p < 0.001$).

An interesting issue is whether the CD28 and B7 deficiencies affect the survival of DN thymocytes. In this study, we investigated the number of cells underlying programmed cell death in the WT, B7-1/2-, and CD28-deficient mice by staining with annexin V. As shown in Fig. 5, B7-1/2- and CD28-deficient DN4 had significantly more annexin $V^+$ cells than the WT counterpart. These results revealed that knocking out B7 and CD28 had similar effects in causing the death of DN4.

### *Targeted mutation of B7 and CD28 induces TCR expression among DN4*

An important feature of the small DN4 T cells is the expression of cell surface pre-TCR complex. To test the effect of costimulation on the up-regulation of TCR$\beta$, we compared DN1–4 from WT, B7-1/2-, and CD28-deficient mice for the expression of TCR. Although a significant portion of $CD44^{high}CD25^{-}$ (DN1) cells expresses TCR$\beta$ on the cell surface, these cells belong to the NKT lineages, as has been reported by others (46). In addition, no $TCR\beta^{high}$ thymocytes were found with the DN2 populations. A small number of DN3 and ~20% of DN4 thymocytes express TCR$\beta$ at significant levels. Interestingly, in the B7KO mice, the overwhelming majority (nearly 80%) of DN4 express TCR$\beta$, while >60% of DN4 in the CD28-deficient mice are $TCR\beta^{high}$ (Fig. 6). Thus, costimulation by B7-1/2 and CD28 genes inhibits the expression of the TCR$\beta$ on DN4.

### *Elimination of cycling thymocytes recapitulates the basic features of DN subsets in mice with targeted mutations of B7-1/2 or CD28*

Given the overall role of B7-CD28 interaction in promoting T cell proliferation and survival (47), it is of great interest to determine whether the altered DN subset distribution and enhanced expression of TCR are a consequence of reduced TCR proliferation and survival. To test this hypothesis, we treated WT C57BL/6 mice with demecolcine, which kills cells undergoing mitosis. As shown in Fig. 7*A*, three consecutive treatments with demecolcine over a 3-day period removed the overwhelming majority of dividing cells within the DN thymocytes. When CD25 and CD44 expression was

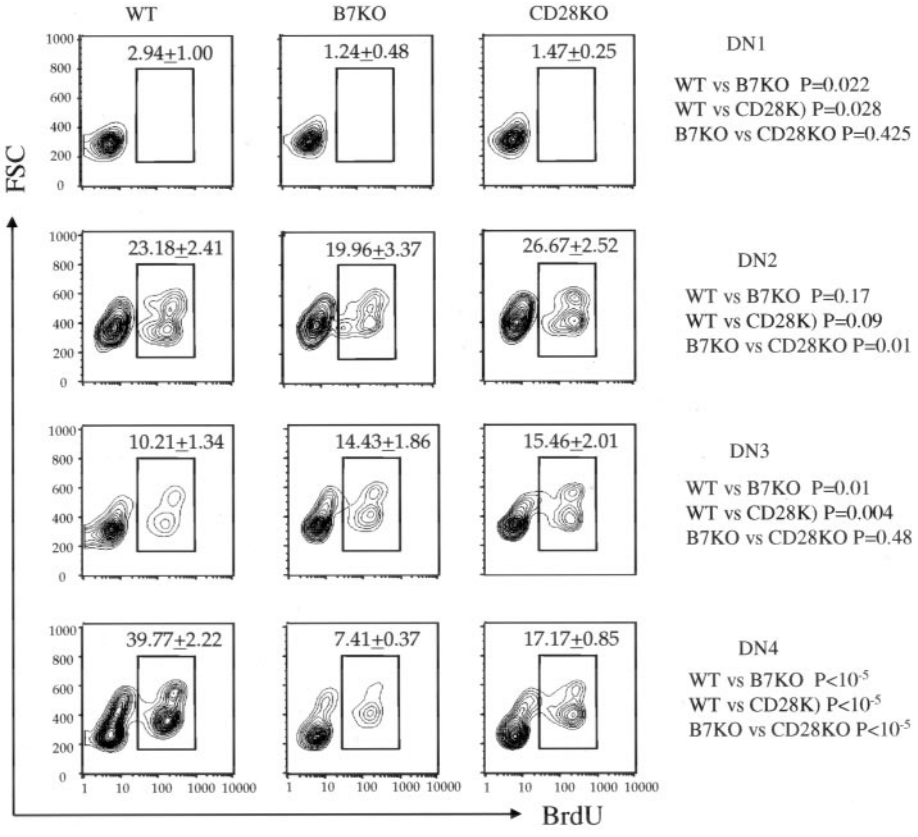


**FIGURE 4.** Target mutations of B7-1 and B7-2 or CD28 decrease the proliferation among DN4 thymocytes. B7KO, CD28KO, and C57BL/6 mice were injected with BrdU i.v. The thymi were harvested at 4 h after BrdU injection and stained with anti-CD4/CD8, anti-CD44, and anti-CD25, and intracellularly stained with anti-BrdU Abs. The numbers in the panels indicate means and SD of the percentage of thymocytes incorporated with BrdU from individual subsets. The *p* values of Student's *t* tests are also provided. Data shown are representative of two independent experiments involving a total of nine mice in each group.

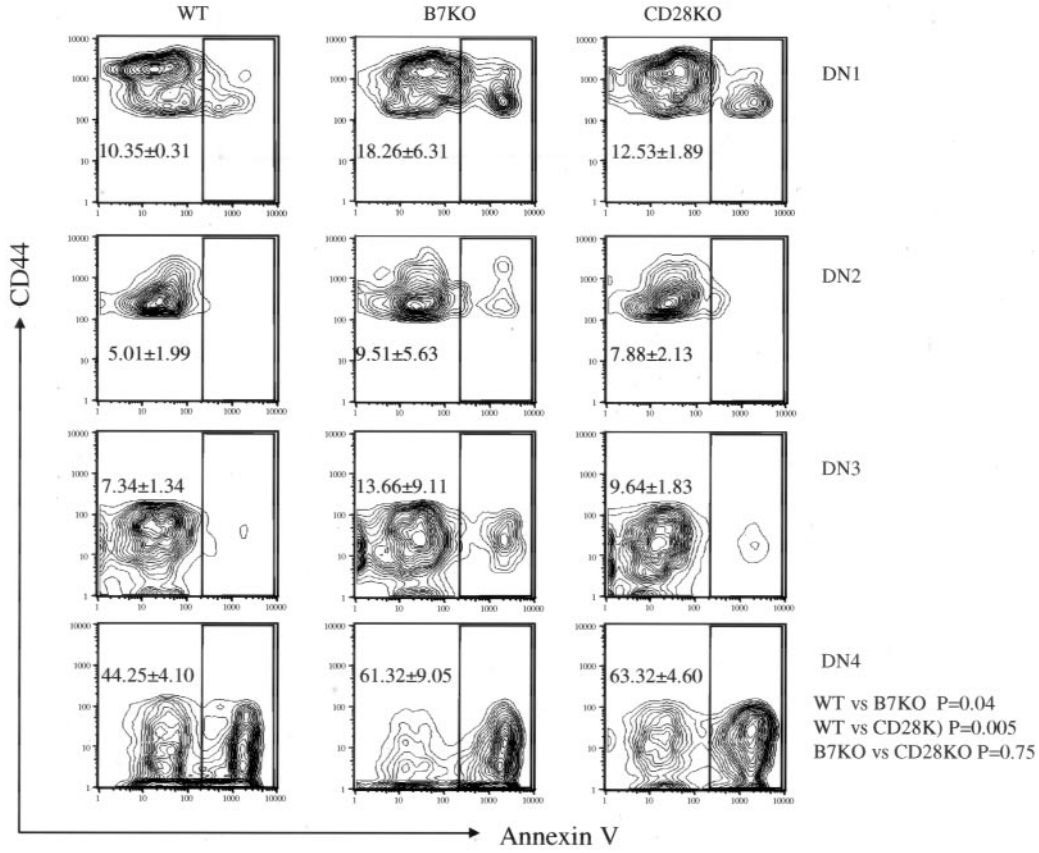


**FIGURE 5.** Target mutation of B7 and CD28 promotes programmed cell death among DN4. B7KO, CD28KO, and C57BL/6 mice were sacrificed at 8–9 wk old, and thymocytes were harvested and stained with anti-CD4/CD8, anti-CD44, and anti-CD25 Abs and annexin V. Data shown are representative of three mice in each group. This experiment has been repeated twice involving seven mice in each group.

analyzed, it became clear that deletion of mitotic thymocytes caused a drastic increase in DN4 and reduction in DN3 (Fig. 7*B*). At the same time, the expression of TCR is significantly increased among DN3 and DN4 in mice that received antimitotic treatments (Fig. 7*C*). Thus, antimitotic treatment recapitulates the two main effects of B7 blockade, although the cellular mechanisms may differ in these two conditions.

*Accumulation of RAG-2 and increased expression of rearranged TCRα in mice with targeted mutation of B7-1/2*

It is well established that expression of both RAG-1 and RAG-2 is critical for transition from DN3 to DN4, as thymocyte development in mice lacking RAG-1 or RAG-2 is blocked at DN3 (25–27). Because both TCR overexpression and increased DN4 can be explained by

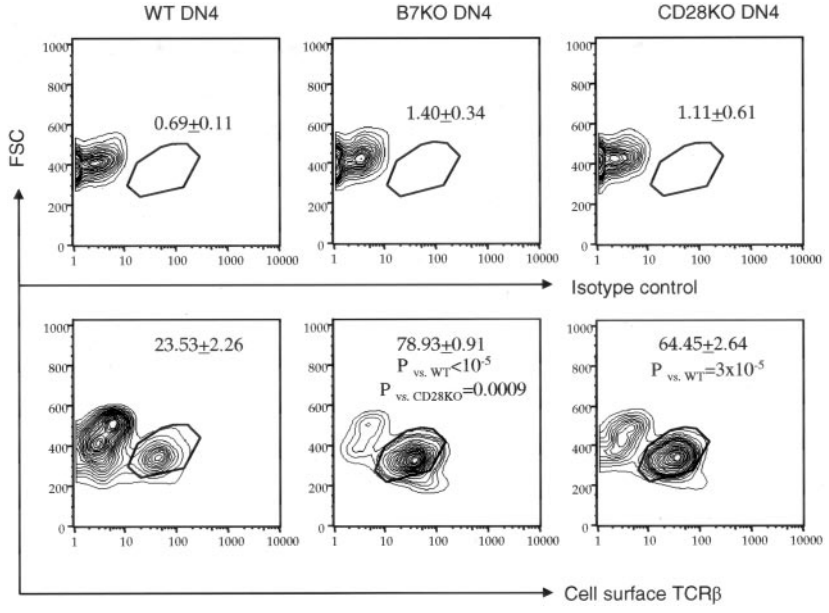


**FIGURE 6.** Target mutation of B7 and CD28 increases TCR expression among DN4. Thymocytes from 8- to 9-wk-old B7KO, CD28KO, and C57BL/6 mice were stained with anti-CD4/CD8, anti-CD44, anti-CD25, anti-TCR $\beta$-chain (*lower panels*), or isotype control Ig (*upper panels*) Abs. Data shown are representative of three mice in each group. This experiment has been repeated twice.

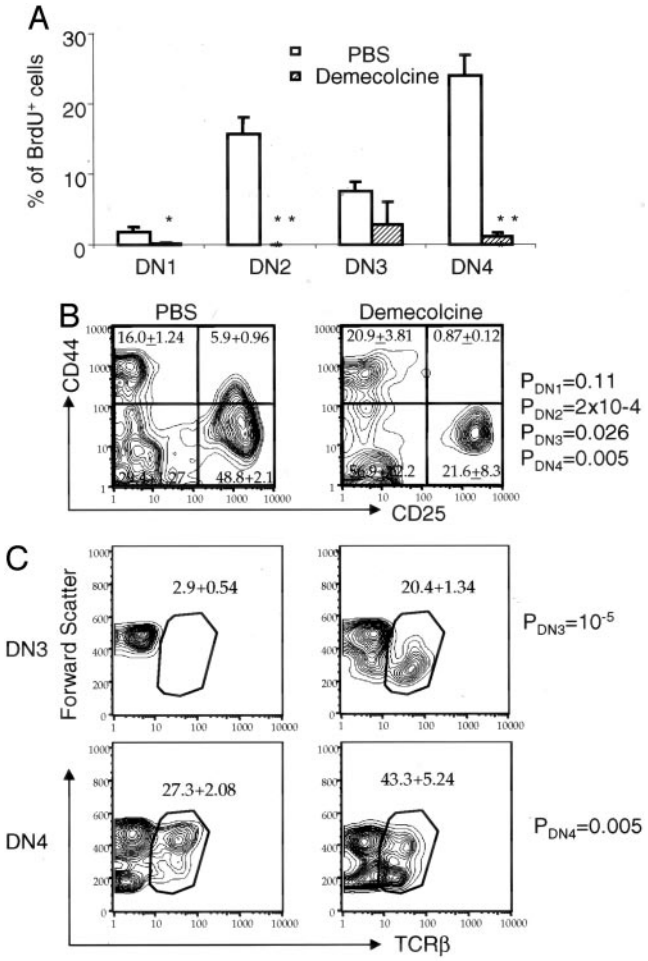


**FIGURE 7.** Antimitotic treatment recapitulates the effect of defective T cell costimulation on DN development. *A*, The efficiency of antimitotic treatment as revealed by reduction in BrdU incorporation. Data shown are means and SD ($n = 4$) of the percentage of $BrdU^+$ T cells within the DN subsets. *B*, Antimitotic treatment increases DN4 while reducing DN2 and DN3. *C*, Antimitotic treatment increases TCR expression on DN3 and DN4 thymocytes. Data shown in *B* and *C* are representative contour graphs from one mouse in groups of four mice. The numbers in the quadrants are means and SD of the percentage of cells in the subset. This experiment has been repeated three times. *, $p < 0.05$; **, $p < 0.01$.

increased RAG activity, we analyzed expression and accumulation of RAG-1/2 mRNA by RT-PCR and RAG-2 protein by Western blot. As shown in Fig. 8, *A* and *B*, purified DN cells from B7KO mice expressed significantly more RAG-2 protein than WT mice. This increase in B7KO DN cells is most likely due to posttranscriptional mechanisms, as the RAG-1/2 mRNA was not increased (Fig. 8*C*). Corresponding to increased RAG-2 protein, real-time RT-PCR revealed a 2- to 3-fold increase in V$\alpha$2 and V$\alpha$4 expression among the DN from B7-deficient mice in comparison with WT mice (Fig. 8*C*).

*Expression of B7-1 and B7-2 in the thymus*

A previous report (48) suggested that expression of B7, as revealed by immunohistochemistry with fusion protein CTLA4Ig, is restricted to the medulla. However, the development of DN takes place in the cortex. To reconcile this apparent inconsistency, we revisited the expression of B7-1 and B7-2 by immunohistochemistry using anti-B7-1 and anti-B7-2 Abs. As shown in Fig. 9, B7-1 expression was observed only in the medulla. Significant levels of B7-2, however, were detected in both cortex and medulla, although the intensity of B7-2 was also higher in the medulla. The difference between current data and the previous work most likely reflects the higher affinity of mAbs than the fusion protein, CTLA4Ig.

*Subset distribution of thymocytes among age-matched WT, $CD28^{-/-}$, and $B7^{-/-}$ mice*

Because the DN4 cells are the immediate precursor for the DP cells, it is possible that abnormal DN development may be associated with altered thymocyte subsets. As shown in Table I, mutations in B7 and CD28 genes resulted in 10–20% increase in $CD4^-CD8^-$ thymocyte and a small, but statistically highly significant decrease in $CD4^+CD8^+$ T cells. The percentage of SP CD4 and CD8 T cells was substantially increased.

## Discussion

The T cell progenitors undergo rapid proliferation and phenotypic transitions before they emerge as the $CD4^+CD8^+$ T cell precursors for positive and negative selection (49). However, the cell surface interactions that guide the first phase of T cell development are still poorly understood. The potential involvement of costimulatory molecules in this phase has not been investigated. In this study, we provide several lines of evidence that demonstrate a critical contribution of costimulatory molecule B7-1/2 and their receptor CD28.

The most clear-cut demonstration of the impact of costimulation on DN development is the alteration of the relative amounts of $CD44^-CD25^+$ (DN3) and $CD44^-CD25^-$ (DN4) cells in mice with inactivated B7-1/2 and CD28. In the WT mice, there are 2–3 times more DN3 than DN4 cells, while B7-deficient mice have 3-fold more DN4 than DN3 cells. Theoretically, there are at least four potential mechanisms that can account for the reduction of DN3 in B7-1/2- and CD28-deficient mice, namely, the proliferation and survival of DN3, a decreased transition from DN2 to DN3, an increased transition from DN3 to DN4, and a reduced rate of transition from DN to DP. Our data effectively ruled out the first possibility, as the proliferation and survival of DN3 were not inhibited by the targeted mutations. A CD28-mediated decrease in DN2 to DN3 transition is not probable, as the DN2 lacks cell surface CD28 expression, while a very small proportion of DN3 expresses CD28.

More rapid transition from DN3 to DN4 is likely to contribute to both a decrease in DN3 and increase in DN4, as these changes cannot be accounted for by death or proliferation in DN3 and DN4. This interpretation is also supported by increased accumulation of RAG-2, which mediates a critical checkpoint between DN3 and DN4. Paradoxically, despite the overall trend of decreased proliferation and survival, the number of total DN and especially DN4 is significantly increased. This can be explained if one assumes that the progression from DN4 to DP is kinetically slower in mice with mutations of B7-1, B7-2, and CD28. This interpretation is consistent with the fact that the DP subset is decreased in the mutant mice. The decrease is statistically highly significant, although not large numerically. However, the decrease caused by delayed DN to DP transition can be more substantial if one considers the decrease in the context of previous works, which shows that costimulation blockade decreases clonal deletion (10, 14). As such, the decrease in DP, resulted from delayed DN to DP progression, may have been counterbalanced by the increase of DP caused by defective clonal deletion. The increase in $CD4^+CD8^-$ and $CD4^-CD8^+$ cells can be attributed directly to abnormal clonal deletion, although altered differentiated DN may have an indirect effect.

Our analysis of proliferation and programmed cell death of the T cell progenitors in the thymus reveals two more points.

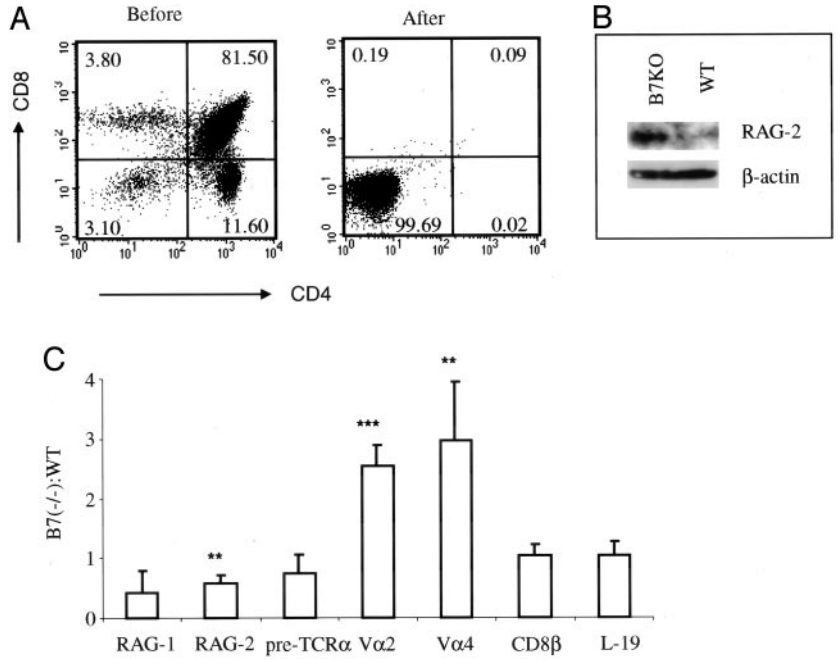


**FIGURE 8.** Increased accumulation of RAG-2 protein and rearranged TCR$\alpha$ in DN population of B7-1/2-deficient mice. Total thymocytes from 8- to 9-wk-old B7KO and C57BL/6 mice were collected, and DN thymocytes were purified by removing cells expressing CD4, CD8, TCR$\gamma\delta$, and I-$A^b$. In each experiment, three thymi per group were combined to isolate DN cells for protein and total RNA extraction. The experiments were repeated twice. *A*, Thymocyte subsets before and after purification. *B*, The RAG-2 protein was detected with Western blot. *C*, The expressions of *RAG-1*, *RAG-2*, *pre-TCR$\alpha$*, TCR *V$\alpha$2* and *V$\alpha$4*, and *CD8 $\beta$* genes were detected with real-time RT-PCR. **, $p < 0.01$; ***, $p < 0.001$. Data shown in this figure have been repeated twice.

First, in parallel to what was described for activation and effector function of mature T cells, the costimulatory molecules B7-1 and B7-2 promote proliferation and survival of immature T cells. Thus, in comparison with WT thymocytes, an increased proportion of DN4 in B7- and CD28-deficient mice was undergoing programmed cell death. Conversely, a substantially reduced percentage of DN4 cells in B7- or CD28-deficient mice incorporated BrdU. Interestingly, in mice with mutations of either B7 or CD28, the proportion of dividing and apoptotic cells appears to have increased among DN2 and DN3 subsets. However, because these two subsets are diminished in the mutant mice, the changes in proportions do not translate into an increased number of cells undergoing division or apoptosis. Differences in apoptosis and proliferation were also noted among the DN1 subset that are equally represented in WT and mutant mice. However, because our study does not differentiate between true DN1 and those that express TCR and CD44 (mostly NKT cells), the significance of the difference in DN1 is unclear at this point.

Second, the function of B7-CD28 interaction in promoting division of DN thymocytes may explain the more rapid DN3 to DN4 transition in mice with mutations of B7 or CD28. This is due to the fact that this transition requires RAG activity restricted to the $G_0/G_1$ phase of the cell cycle (50, 51). Indeed, RAG-2 protein is accumulated at $G_0/G_1$, and its expression level decreases rapidly at the $G_1$-S transition of the cell cycle by cytoplasmic sequestration and ubiquitin-dependent degradation (52). Our analysis of RAG-2 protein and TCR gene expression clearly demonstrated that DN from B7-deficient mice have increased accumulation of RAG-2 protein and increased expression of rearranged TCR genes. The link between cell division and DN3-DN4 transition is supported by

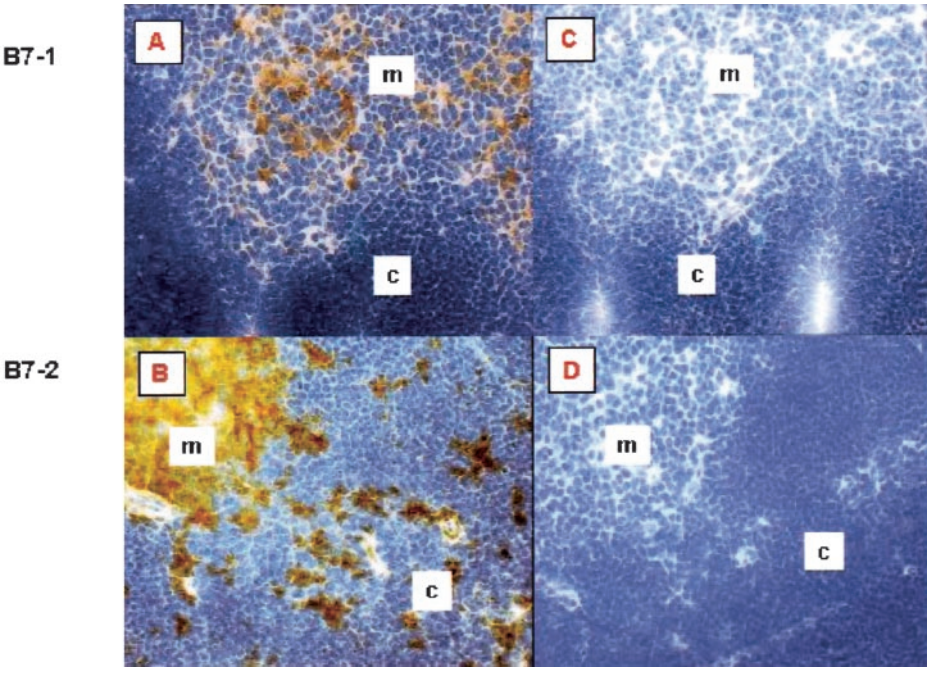


**FIGURE 9.** Expression of B7-1 and B7-2 in the thymus. Frozen thymic sections were stained with either anti-B7-1 (A) and anti-B7-2 (B), followed by second-step reagents, biotinylated goat anti-hamster (A and C), or goat anti-rat (B and D) IgG. The cortex (c) and medulla (m) are marked.

Table I. *Impact of targeted mutations of B7-1/2 and CD28 on the subsets of T cells in the thymus*[a]

| Mice | Subsets | | | | |
|---|---|---|---|---|---|
| | No. ($\times 10^{-6}$) | $CD4^-CD8^-$ | $CD4^+CD8^+$ | $CD4^-CD8^+$ | $CD4^+CD8^-$ |
| WT | 88.0 ± 24.28 | 3.02 ± 0.16 | 83.62 ± 0.92 | 3.37 ± 0.35 | 9.99 ± 0.5 |
| $B7^{-/-}$ | 69.80 ± 7.69 | 3.64 ± 0.35 | 77.32 ± 1.26 | 4.43 ± 0.39 | 14.62 ± 0.90 |
| $CD28^{-/-}$ | 59.00 ± 16.76 | 3.32 ± 0.17 | 75.53 ± 1.7 | 4.60 ± 0.63 | 16.55 ± 1.40 |
| $P_{87KO/WT}$ | 0.15 | 0.007 | 0.00002 | 0.002 | 0.0001 |
| $P_{CD28KO/WT}$ | 0.06 | 0.02 | 0.00001 | 0.005 | 0.00001 |

[a] Thymocytes from age- and sex-matched WT, $CD28^{-/-}$ or B7-$1^{-/-}$ B7-$2^{-/-}$ mice were analyzed by flow cytometry. Data shown are means and SD from a representative experiment involving five mice per group. This experiment has been repeated three (total and DN) or two (other subsets) times.

our data that antimitotic treatment results in changes of DN thymocytes that are not unlike those observed in B7- and CD28-deficient mice, including the ratio of DN3/DN4 and cell surface TCR. Because more cells in DN4 incorporate BrdU than those in DN3, the increased cell number in DN4 during antimitotic treatment is most likely due to a combination of two defects: an increased transition from DN3 and a delayed transition out of DN4.

The expression of B7-2 in the cortex is consistent with the idea that B7-CD28 interaction is directly involved in differentiation in DN. This is further strengthened by our observation that most of the effects in DN division and survival are seen in DN4 that express high levels of CD28. However, due to the genetic nature of the current study, it is theoretically possible that the observed effect is not an indirect consequence of such interaction. Another interesting issue is the identity of B7 receptors that may be involved in the early stage of T cell development. Although mutations of B7 and CD28 have qualitatively similar phenotypes, mutations of B7-1 and B7-2 have significantly more severe effect than those of CD28. A natural question is whether this is attributable to CTLA4, the other known B7 receptor. Although CTLA4 protein is expressed early among DN2 cells, targeted mutation of CTLA4 does not affect DN subset composition. It is therefore unlikely that CTLA4 is involved in the differentiation of DN. It is possible that another unidentified B7 receptor (40) may participate in this process. Regardless of what the additional receptor may be, our results extended the functional spectrum of T cells that are modulated by the costimulatory pathway.

## Acknowledgments

We thank Lynde Shaw for editorial assistance and Lijie Yin for mouse breeding.

## References

1. Norton, S. D., L. Zuckerman, K. B. Urdahl, R. Shefner, J. Miller, and M. K. Jenkins. 1992. The CD28 ligand, B7, enhances IL-2 production by providing a costimulatory signal to T cells. *J. Immunol. 149:1556*.
2. Harding, F. A., and J. P. Allison. 1993. CD28–B7 interactions allow the induction of $CD8^+$ cytotoxic T lymphocytes in the absence of exogenous help. *J. Exp. Med. 177:1791*.
3. Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, and J. P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature 356:607*.
4. Ramarathinam, L., M. Castle, Y. Wu, and Y. Liu. 1994. T cell costimulation by B7/BB1 induces CD8 T cell-dependent tumor rejection: an important role of B7/BB1 in the induction, recruitment, and effector function of antitumor T cells. *J. Exp. Med. 179:1205*.
5. Zheng, P., S. Sarma, Y. Guo, and Y. Liu. 1999. Two mechanisms for tumor evasion of preexisting cytotoxic T-cell responses: lessons from recurrent tumors. *Cancer Res. 59:3461*.
6. Sarma, S., Y. Guo, Y. Guilloux, C. Lee, X. F. Bai, and Y. Liu. 1999. Cytotoxic T lymphocytes to an unmutated tumor rejection antigen P1A: normal development but restrained effector function in vivo. *J. Exp. Med. 189:811*.
7. Bai, X. F., J. Bender, J. Liu, H. Zhang, Y. Wang, O. Li, P. Du, P. Zheng, and Y. Liu. 2001. Local costimulation reinvigorates tumor-specific cytolytic T lymphocytes for experimental therapy in mice with large tumor burdens. *J. Immunol. 167:3936*.
8. Allison, J., L. A. Stephens, T. W. Kay, C. Kurts, W. R. Heath, J. F. Miller, and M. F. Krummel. 1998. The threshold for autoimmune T cell killing is influenced by B7-1. *Eur. J. Immunol. 28:949*.
9. Chang, T. T., C. Jabs, R. A. Sobel, V. K. Kuchroo, and A. H. Sharpe. 1999. Studies in B7-deficient mice reveal a critical role for B7 costimulation in both induction and effector phases of experimental autoimmune encephalomyelitis. *J. Exp. Med. 190:733*.
10. Gao, J.-X., H. Zhang, X. F. Bai, J. Wen, X. Zheng, J. Liu, P. Zheng, and Y. Liu. 2002. Peripheral blockade of B7-1 and B7-2 inhibits clonal deletion of highly pathogenic autoreactive T cells. *J. Exp. Med. 195:959*.
11. Noel, P. J., M. L. Alegre, S. L. Reiner, and C. B. Thompson. 1998. Impaired negative selection in CD28-deficient mice. *Cell. Immunol. 187:131*.
12. Kishimoto, H., and J. Sprent. 1999. Several different cell surface molecules control negative selection of medullary thymocytes. *J. Exp. Med. 190:65*.
13. Li, R., and D. M. Page. 2001. Requirement for a complex array of costimulators in the negative selection of autoreactive thymocytes in vivo. *J. Immunol. 166:6050*.
14. Buhlmann, J. E., S. K. Elkin, and A. H. Sharpe. 2003. A role for the B7-1/B7-2:CD28/CTLA-4 pathway during negative selection. *J. Immunol. 170:5421*.
15. Cibotti, R., J. A. Punt, K. S. Dash, S. O. Sharrow, and A. Singer. 1997. Surface molecules that drive T cell development in vitro in the absence of thymic epithelium and in the absence of lineage-specific signals. *Immunity 6:245*.
16. Groves, T., M. Parsons, N. G. Miyamoto, and C. J. Guidos. 1997. TCR engagement of $CD4^+CD8^+$ thymocytes in vitro induces early aspects of positive selection, but not apoptosis. *J. Immunol. 158:65*.
17. Salomon, B., D. J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J. A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the $CD4^+CD25^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity 12:431*.
18. Salomon, B., and J. A. Bluestone. 2001. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annu. Rev. Immunol. 19:225*.
19. Bour-Jordan, H., and J. A. Blueston. 2002. CD28 function: a balance of costimulatory and regulatory signals. *J. Clin. Immunol. 22:1*.
20. Baecher-Allan, C., J. A. Brown, G. J. Freeman, and D. A. Hafler. 2001. $CD4^+CD25^{high}$ regulatory cells in human peripheral blood. *J. Immunol. 167:1245*.
21. Michie, A. M., J. R. Carlyle, T. M. Schmitt, B. Ljutic, S. K. Cho, Q. Fong, and J. C. Zuniga-Pflucker. 2000. Clonal characterization of a bipotent T cell and NK cell progenitor in the mouse fetal thymus. *J. Immunol. 164:1730*.
22. Baird, A. M., R. M. Gerstein, and L. J. Berg. 1999. The role of cytokine receptor signaling in lymphocyte development. *Curr. Opin. Immunol. 11:157*.
23. Moore, T. A., U. von Freeden-Jeffry, R. Murray, and A. Zlotnik. 1996. Inhibition of γδ T cell development and early thymocyte maturation in IL-$7^{-/-}$ mice. *J. Immunol. 157:2366*.
24. Waskow, C., and H. R. Rodewald. 2002. Lymphocyte development in neonatal and adult c-Kit-deficient (c-KitW/W) mice. *Adv. Exp. Med. Biol. 512:1*.
25. Rodewald, H. R., M. Ogawa, C. Haller, C. Waskow, and J. P. DiSanto. 1997. Pro-thymocyte expansion by c-*kit* and the common cytokine receptor γ chain is essential for repertoire formation. *Immunity 6:265*.
26. Rodewald, H. R., K. Kretzschmar, W. Swat, and S. Takeda. 1995. Intrathymically expressed c-*kit* ligand (stem cell factor) is a major factor driving expansion of very immature thymocytes in vivo. *Immunity 3:313*.
27. Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa, and V. E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell 68:869*.
28. Shinkai, Y., G. Rathbun, K. P. Lam, E. M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A. M. Stall, et al. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell 68:855*.
29. Godfrey, D. I., J. Kennedy, P. Mombaerts, S. Tonegawa, and A. Zlotnik. 1994. Onset of TCR-β gene rearrangement and role of TCR-β expression during $CD3^-CD4^-CD8^-$ thymocyte differentiation. *J. Immunol. 152:4783*.
30. Mombaerts, P., A. R. Clarke, M. A. Rudnicki, J. Iacomini, S. Itohara, J. J. Lafaille, L. Wang, Y. Ichikawa, R. Jaenisch, M. L. Hooper, et al. 1992. Mutations in T-cell antigen receptor genes α and β block thymocyte development at different stages. *Nature 360:225*.
31. Fehling, H. J., A. Krotkova, C. Saint-Ruf, and H. von Boehmer. 1995. Crucial role of the pre-T-cell receptor α gene in development of αβ but not γδ T cells. *Nature 375:795*.
32. Borriello, F., M. P. Sethna, S. D. Boyd, A. N. Schweitzer, E. A. Tivol, D. Jacoby, T. B. Strom, E. M. Simpson, G. J. Freeman, and A. H. Sharpe. 1997. B7-1 and B7-2 have overlapping, critical roles in immunoglobulin class switching and germinal center formation. *Immunity 6:303*.

33. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science 261:609*.
34. Bachmann, M. F., A. Gallimore, E. Jones, B. Ecabert, H. Acha-Orbea, and M. Kopf. 2001. Normal pathogen-specific immune responses mounted by CTLA-4-deficient T cells: a paradigm reconsidered. *Eur. J. Immunol. 31:450*.
35. Wurch, A., J. Biro, I. Falk, H. Mossmann, and K. Eichmann. 1999. Reduced generation but efficient TCR $\beta$-chain selection of $CD4^+8^+$ double-positive thymocytes in mice with compromised CD3 complex signaling. *J. Immunol. 162:2741*.
36. Zheng, X., J. X. Gao, H. Zhang, T. L. Geiger, Y. Liu, and P. Zheng. 2002. Clonal deletion of simian virus 40 large T antigen-specific T cells in the transgenic adenocarcinoma of mouse prostate mice: an important role for clonal deletion in shaping the repertoire of T cells specific for antigens overexpressed in solid tumors. *J. Immunol. 169:4761*.
37. Wu, Y., Y. Guo, and Y. Liu. 1993. A major costimulatory molecule on antigen-presenting cells, CTLA4 ligand A, is distinct from B7. *J. Exp. Med. 178:1789*.
38. Hathcock, K. S., G. Laszlo, H. B. Dickler, J. Bradshaw, P. Linsley, and R. J. Hodes. 1993. Identification of an alternative CTLA-4 ligand costimulatory for T cell activation. *Science 262:905*.
39. Yamada, A., K. Kishimoto, V. M. Dong, M. Sho, A. D. Salama, N. G. Anosova, G. Benichou, D. A. Mandelbrot, A. H. Sharpe, L. A. Turka, et al. 2001. CD28-independent costimulation of T cells in alloimmune responses. *J. Immunol. 167:140*.
40. Mandelbrot, D. A., M. A. Oosterwegel, K. Shimizu, A. Yamada, G. J. Freeman, R. N. Mitchell, M. H. Sayegh, and A. H. Sharpe. 2001. B7-dependent T-cell costimulation in mice lacking CD28 and CTLA4. *J. Clin. Invest. 107:881*.
41. Chambers, C. A., D. Cado, T. Truong, and J. P. Allison. 1997. Thymocyte development is normal in CTLA-4-deficient mice. *Proc. Natl. Acad. Sci. USA 94:9296*.
42. Levelt, C. N., and K. Eichmann. 1995. Receptors and signals in early thymic selection. *Immunity 3:667*.
43. Fehling, H. J., and H. von Boehmer. 1997. Early $\alpha\beta$ T cell development in the thymus of normal and genetically altered mice. *Curr. Opin. Immunol. 9:263*.
44. Penit, C., B. Lucas, and F. Vasseur. 1995. Cell expansion and growth arrest phases during the transition from precursor ($CD4^-8^-$) to immature ($CD4^+8^+$) thymocytes in normal and genetically modified mice. *J. Immunol. 154:5103*.
45. Vasseur, F., A. Le Campion, and C. Penit. 2001. Scheduled kinetics of cell proliferation and phenotypic changes during immature thymocyte generation. *Eur. J. Immunol. 31:3038*.
46. MacDonald, H. R. 2002. Development and selection of NKT cells. *Curr. Opin. Immunol. 14:250*.
47. Boise, L. H., A. J. Minn, P. J. Noel, C. H. June, M. A. Accavitti, T. Lindsten, and C. B. Thompson. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-$x_L$. *Immunity 3:87*.
48. Degermann, S., C. D. Surh, L. H. Glimcher, J. Sprent, and D. Lo. 1994. B7 expression on thymic medullary epithelium correlates with epithelium-mediated deletion of $V\beta5^+$ thymocytes. *J. Immunol. 152:3254*.
49. Huesmann, M., B. Scott, P. Kisielow, and H. von Boehmer. 1991. Kinetics and efficacy of positive selection in the thymus of normal and T cell receptor transgenic mice. *Cell 66:533*.
50. Desiderio, S., W. C. Lin, and Z. Li. 1996. The cell cycle and V(D)J recombination. *Curr. Top. Microbiol. Immunol. 217:45*.
51. Lee, J., and S. Desiderio. 1999. Cyclin A/CDK2 regulates V(D)J recombination by coordinating RAG-2 accumulation and DNA repair. *Immunity 11:771*.
52. Mizuta, R., M. Mizuta, S. Araki, and D. Kitamura. 2002. RAG2 is down-regulated by cytoplasmic sequestration and ubiquitin-dependent degradation. *J. Biol. Chem. 277:41423*.

# THE B7-CD28 INTERACTION IS CRITICAL IN THE DEVELOPMENT AND EFFECTOR FUNCTION OF NKT CELL

Xincheng Zheng*, Huiming Zhang*, Chyung-Ru Wang#, Yang Liu* and Pan Zheng*

From

*Division of Cancer Immunology, Department of Pathology and Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210

#Gwen Knapp Center, University of Chicago, Chicago, IL.

Correspondence should be addressed to:

Pan Zheng, Division of Cancer Immunology, Department of Pathology and Comprehensive Cancer Center, The Ohio State University Medical Center

129 Hamilton Hall, 1645 Neil Avenue, Columbus, OH 43210

Ph: 614-292-2003, FAX: 614-688-8152, E-mail: zheng-1@medctr.osu.edu

This work was supported by grants from the Department of Defense (DAMD17-03-1-0013 to PZ), National Institute of Health (CA69091, and CA58033 to YL), and by the Ohio State University Comprehensive Cancer Center.

**Abstract**

Natural Killer T (NKT) cell is a unique subset of T cells characterized by expressing both invariant TCR and various NK linage markers, and is actively involved in viral infection, autoimmunity and tumor immunity. NKT cell developed in thymus and deviated from mainstream of conventional T cell development at CD4CD8 DP stage. In addition to their functions in the induction and effecter phase of T cells, our recent studies have demonstrated that the costimulatory molecules also contribute to the developmental process of T cell at immature, DN stage. However, the contribution of costimulatory molecules to the development and function of NKT cells has never been studied before. Here we report that the development of NKT cells are defect in the mice with targeted mutations of B7-1/2 and CD28, the percentage of $TCR_{\beta}^{+}NK1.1^{+}$, as well as $TCR_{\beta}^{+}$ α-Galcer/CD1d $^{+}$ (iVα14 NKT) cells population are significantly reduced in the thymus, spleen and liver. More ever, the constitution of iVα14 NKT cells is changed in B7 and CD28 deficient mice, as shown by decreased $CD4^{-}NK1.1^{+}$ subset, while increased $CD4^{+}NK1.1^{-}$ subset. In consistent with these, the mice with target mutation of costimulatory molecules has defect NKT cell function. B7 and CD28 deficient mice develop much less sever ConA induced hepatitis, which is known mediated by NKT cells. Taken together, our results demonstrate that development and function of NKT cell is subject to modulation by the costimulatory pathway.

## Introduction

Natural Killer T (NKT) cell is a unique subset of T cells expressing both TCR, as well as other typical NK linage receptors (1-5). However, unlike NK cells, NKT cells mainly develop in the thymus. In contrast to conventional T cells, NKT cells respond to antigen presented by the nonpolymorphic major histocompatibility complex (MHC) Class I-like molecule CD1d and express an extremely limited T cell repertoire (3-5). Their TCR is composed almost exclusively of Vα14/Jα18 in mouse and Vα24/Jα18 in human. In contrast to typical $CD8^+$ Class I restricted T cells, NKT cells are either $CD4^+$ or $CD4^-CD8^-$ (DN), and in mice the TCR receptor α chain is paired with Vβ8.2, Vβ7, or Vβ2 TCRβ chain that bind lipids, glycolipids, or highly hydrophobic peptides presented by CD1D molecules (4, 5). Upon stimulated through their TCR, the immediate response of NKT cells is to produce substantial amounts of cytokines especially IL-4 and IFN-r. So they are not quiet fit the Th1/Th2 classifications (6, 7). Although the exact function of NKT cell is largely unknown, emerging evidences have demonstrated that it involves in infection (8), tumor (9) and autoimmune disease (10) by either direct cytotoxicity or function as a regulatory cell by secreting several cytokines.

The developmental relationship of NKT to conventional T cells has been controversial. Two models have been proposed for NKT cell development(11, 12). NKT cells might derive from distinct precursor that undergoes lineage commitment independently of TCR-ligand interactions (pre-committed model). Alternatively, they may develop as a byproduct of conventional T cell development at certain stage, depending on the ligand they recongnize (TCR-instruction model). Recently, by mature NKT cells developed following intrathymic injection of CD4+CD8+ cells, Gapin et al.

showed that NKT cells can developed in the thymus from double positive stage of conventional thymocyte development (13). Moreover, rather than thymic epithelial cells, the positive and negative selections of NKT cells rely on CD1d presented by hematopoietic derived CD4+CD8+ thymocyte and dendritic cells respectively (13, 14). The details of this selective process are still largely unknown. An intermediate, semi-mature $CD4^{+}NK1.1^{-}$ stage has been proposed before NKT cells finally develop into $NK1.1^{+}$ cells that are either $CD4^{+}$ or double negative (15-17). Compared with limited data suggesting costimulatory molecules may involve in positive selection by modulating the strength of TCR signaling (18), several lines of evidences have shown that costimulatory molecules are critical for negative selections of conventional T cells encountering self-antigen presented by classical MHC. Either blockade B7-1/2 in thymic organ culture or perinatally blocking B7-1/2 with mAbs in vivo could significantly reduce the efficacy of clonal deletion of antigen specific T cells(19, 20). Moreover, the data from our group also demonstrated that B7 and CD28 may function as a critical signal to promote proliferation and differentiation of DN T cells in the thymus (21). The deficiencies of B7 or CD28 reduce proliferation and increase programmed cell death of late-stage DN T cells (DN4, $CD44^{-}CD25^{-}$). This established a role of T cell costimulation in the development of early T-cell progenitors. As a unique lineage of T cell branched away from main stream double positive thymocytes, the B7-CD28 interaction may also modulate the development of NKT cells. However, this has never been tested before.

Here we show that the development of NKT cells are defect in the mice with targeted mutations of B7-1 and B7-2, the population of $TCR\beta^{+}NK1.1^{+}$, especially the CD1d restricted $TCR\beta^{+}NK1.1^{+}$ cells, are significantly decreased in the thymus, spleen

and liver of B7-1/2 and CD28 mutant mice. Most interestingly, in $TCR\beta^{+}CD1d\text{-}\alpha\text{-}Galcer^{+}$ population of adult mice, target mutation of B7-1 and B7-2 will significantly decrease $CD4^{-}NK1.1^{+}$ subpopulation, while increase $CD4^{+}NK1.1^{-}$ subsets. Qualitatively similar effects were observed in mice with targeted mutation of CD28. Therefore, in addition to modulate the development of iVα14 NKT cells, the costimulatory signaling is also necessary for optimal differentiation of NKT in central and periphery. Further more, ConA injection can induce less severe hepatitic injury in the mice with target mutation of costimulatory molecules than it in wild type mice, indicating NKT cell function is also defect in these mice. Taken together, our results demonstrate that development and function of NKT cell are subjects to modulation by the costimulatory pathway.

## Materials and Methods

### Experimental animals

Wild-type C57BL/6j, B7-1/2 double knockout and CD28 knockout mice on C57BL/6j background were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animals were maintained in the University Laboratory Animal Research Facility at the Ohio State University under specific-pathogen-free conditions.

### Cell Preparation, antibodies and Flow cytometry

Single cell suspensions from thymus and spleen were prepared by mechanical disruption in cold serum free RPMI 1640 medium. The liver was perfused with 1xPBS via portal vein until the color changed to pale. Then the liver was gently dissociated and incubated with 1mg/ml Collagenase type IV (Sigma, C5138) in 10mM Hepes-NaOH buffer (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$, pH 7.4) for 1 h at 37°C. The whole materials were passed through the syringe for several times to get single cell suspension. The hepatitic lymphocytes can be recovered with 40% vs. 60% Percoll centrifuge. Red blood cells from single cell suspensions were removed by brief hypotonic lysis before cell surface staining.

Both cell surface markers and intracellular staining were analyzed by flow cytometry (Becton Dickinson, Mountain View, CA). The fluorescence conjugated antibodies anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-TCR β chain (H57-597), anti-CD44 (IM7), anti-CD25 (PC61) and anti-NK1.1 (PK136) were purchased from BD

PharMingen (San Diego, CA, USA). PE-conjugated α-GalCer loaded CD1d tetramer was kindly provided by Dr. Chyung-Ru Wang (University of Chicago, Chicago, IL).

**Induction of ConA induced hepatitis**

ConA (Sigma, C0412) was dissolved in pyrogen-free PBS and i.v. injected to mice through the tail vein at a dose of 20mg/kg. Sera from individual mice were obtained 16 h after ConA injection. Serum aminotransferase [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] activities were measured by the standard photometric method using Hitachi type 911 automatic analyzer (Tokyo).

**Histological Examination**

The livers from Con A treated mice were harvested after 16 h, and then were fixed in 10% formalin; embedded in paraffin, sectioned and stained with hematoxylin and eosin for histological examination. Specimens were examined under a light microscope. 3-4 images were collected each section and the injured area was measured with MCID Analysis 7.0 (Imaging Research Inc., Ontario, Canada).

**Statistical Analysis**

Data were statistically analyzed with two-tailed student T-test. $P < 0.05$ is significant and $p < 0.01$ is highly significant.

## Results

### The reduced TCR $\beta^+NK1.1^+$ NKT cells in B7-1/2 and CD28 deficient mice

In order to further illustrate the effect of costimulatory signal on NKT development, we used typical NKT cell markers and compared the percentages of NKT cells in the central and peripheral lymphoid organs of B7-1/2, CD28 mutant mice with age and sex matched C57BL/6 mice. As shown in Fig. 3.1A, TCR $\beta^+NK1.1^+$ cells is about 1% in total thymocytes, and 4.9% and 3.66% in the DN and $CD4^+$ subsets from WT mice respectively. TCR $\beta^+NK1.1^+$ cell has been used as typical markers for NKT cell in certain mouse strains. The expressions of TCR β and NK1.1 are at the intermediate level, compared with T cell and NK cell counterparts, which have been demonstrated before (4). However, they are decreased almost 3-5 fold in the thymus of B7-1/2 mutant mice, as shown in total thymocytes, DN subpopulation and CD4 subset as well. The mutation of CD28 has water even more significant effect on total and $CD4^+$ thymocytes. To further substantiate these observations, we also compare NKT cells in spleen and liver from mice deficient for B7-1/2 and CD28 with WT C57BL/6 controls. As shown in Fig. 3.1B, TCR $\beta^+NK1.1^+$ cells were decreased to 1.56% and 1.27% in total splenocytes from B7-1/2 and CD28 mutant mice respectively, compared with 1.86% in WT mice. If look at $CD4^+$ splenocytes, this population is reduced to 1.54% and 1.11% from B7-1/2 and CD28 mutant mice, compared with 3.15% in WT mice. However, the difference in DN lymphocytes is undistinguishable, and the more sever pattern in CD28 mutant mice can only be seen on total splenocytes. Surprisingly, The TCR $\beta^-NK1.1^+$ (NK) cells are also decreased in total splenocytes and DN splenocytes, from 3% to 2% and 5% to 3% respectively. Therefore, the costimulatory molecules are not only modulate TCR

signaling, but also down-regulate NK1.1 expression. To our knowledge, this has never been reported before. Because the fold of NKT cell reduction is almost the same as it does on NK cell, we cannot exclude the possibility that the decreased NKT cells in total and CD4 T cells in B7-1/2 and CD28 deficiency mice are due to the general down-regulation of NK1.1 receptors.

Liver is a very important peripheral lymphoid organ for NKT cells (22-24), the frequency of NKT cells in liver is much higher than those in other lymphoid organs like spleen. As shown in Fig. 3.1C, more than 5% lymphocytes in total and CD4 T cells are TCR $\beta^{+}NK1.1^{+}$ cells. Except about 50% reduction in CD4 T cells could be detected in CD28 mutant mice, this population was not significantly changed in total and DN lymphocytes of B7-1/2 and CD28 deficient mice. Therefore, the reduction in liver seems not as striking as in thymus. As noted in the figure, the differences between B7 and CD28 are apparently not consistent regarding individual subsets in different organs. Thus, the mutation of CD28 has little, if there is any, more effect on reduction of NKT population. Again, similar with what we found in total splenocytes, the reductions of NK cells are still significant in total (but not DN) liver lymphocytes in B7-1/2 and CD28 deficient mice.

While some NKT cells are CD1d independent, most of NKT cells recognize glycolipid presented by MHC class I-like molecule CD1d, and this population is referred to CD1d-restricted NKT bearing the invariant Vα14 rearrangement (also called iVα14 NKT cells) (25, 26) . Although the endogenous antigen for CD1d is still unclear, a synthetic glycolipid antigen derived from marine sponges called alpha-galactosyl ceramide (α-GalCer), can be recognized by NKT and potently stimulates NKT cells via

TCR when presented by CD1d (27). Recently, the development of fluorescence conjugated α-GalCer-CD1d tetramer reagent provides a unique technique to identify iVα14 NKT cells in vivo (28, 29). In this study, we applied α-GalCer-CD1d tetramer approach to further characterize the TCR $\beta^+NK1.1^+$ cells in B7-1/2 and CD28 deficient mice. As shown in Fig.3.2, when TCR $\beta^+NK1.1^+$ cells were gated and then look at the specificity for α-GalCer-CD1d tetramer and the expression of CD44. In consistent with their activated phenotype, the majority of TCR $\beta^+NK1.1^+$ cells in thymus are -GalCer-CD1d tetramer and CD44 double positive (CD1d restricted iVα14 NKT cells), and this subsets are 56% in spleen and 36% in liver. In addition to the reduction of total TCR $\beta^+NK1.1^+$ cells, CD1d restricted iVα14 NKT cells are further decreased in thymus and spleen. Particularly, even the percentage of TCR $\beta^+NK1.1^+$ cells were not change in total lymphocytes in liver, CD1d restricted iVα14 NKT cells are significantly decreased. The effects are qualitatively and quantitatively same between B7-1/2 and CD28 deficient mice in this analysis. Therefore, the most significant effect in the scenario of costimulatory pathway deficient is on CD1d restricted iVα14 NKT cells.

**The down-regulation of NK1.1 in $TCR^+$α-GalCer/$CD1d^+$ NKT cells in costimulatory molecules deficient mice**

Given the fact that some of $TCR^+NK1.1^+$ NKT cells are CD1d independent and conventional T cells can upregulate NK1.1 expression upon activation (30, 31), the phenotype, $TCR^+NK1.1^+$, which has traditionally been used to identify NKT cells, may not well represent iVα14 NKT cells(17, 32). Thus, TCR combined with α-GalCer/CD1d tetramer is apparently a more reliable reagent that clearly and specifically defines CD1d-

restricted iVα14 NKT cells (17, 32, 33). To further characterize the effect of costimulatory molecules on iVα14 NKT cells development and activation, we directly gated on $TCR^+$α-GalCer/$Cd1d^+$ population and look at the CD4, CD44 and NK1.1 expression patterns.

As shown in Fig.3.3A, in consistent with what we found when traditional NKT cells marker $TCR^+NK1.1^+$ was used, $TCR^+$α-GalCer/$Cd1d^+$ cells were significantly reduced in total lymphocytes from both thymus and spleen of B7-1/2 deficient mice, again qualitative similar but quantitatively more significant reduction could be detected in CD28 deficient mice. However, the reduction is not striking in total lymphocytes from liver. If we looked further on the phenotype of iVα14 NKT cells, we found that all these cells are $CD44^+$, appeared a matured and activated status (Fig. 3.3B). Surprisingly, in B7-1/2 and CD28 deficient mice, the $NK1.1^+$ subset from $TCR^+$α-GalCer/$Cd1d^+$ cells are significantly reduced and correspondingly, $NK1.1^-$ subsets were significantly increased about 4 folds in thymus, and 2 folds in spleen and liver (Fig. 3.3C). When $TCR^+$α-GalCer/$Cd1d^+$ were gated and looked at the constitution of iVα14 NKT by the expression of CD4 and NK1.1, interestingly, there are 2-fold reduction of $NK1.1^+CD4^-$ subset in all lymphoid organs, and 2-fold increase of $NK1.1^-CD4^+$ subset in spleen and liver, while 5-fold increase in thymus of mutant mice. The quantity and quality are the same between B7-1/2 and CD28 deficient mice. Therefore, in addition to the developmental defect on iVα14 NKT cells in B7-1/2 and CD28 deficient mice, which was demonstrated by reduced percentage of $TCR^+$α-GalCer/$Cd1d^+$ cells, the differentiation of NKT is also abnormal in mutant mice, as shown by different constitution in iVα14 NKT cells. The

changed ratios between $NK1.1^{+}CD4^{-}$ and $NK1.1^{-}CD4^{+}$ subsets implied functional differences in B7-1/2 and CD28 deficient mice.

**The defect NKT function in costimulatory molecules deficient mice**

As we mentioned before, NKT cells have an unusual tissue distribution. In the peripheral lymphoid organs, they are abundant in the liver, but are relatively rare in spleen and lymph nodes (4, 34). This indicated their crucial function in liver. Although the physiological function of NKT cells in liver remains unclear, several publications demonstrated the contribution of liver NKT cells to a Con-A induced murine hepatitis model (34, 35). It has been shown that Con-A administration induced hepatitis is mediated by FasL expressed NKT cells, while CD1d-deficient mice lacking NKT cells were highly resistant to Con-A induced hepatitis (34).

To explore the possible defect NKT function due to reduction of iVα14 NKT cells in B7 and CD28 deficient mice, we i.v. injected Con A into B7-1/2, CD28 deficient mice and WT C57BL/6 mice and measured serum AST and ALT levels and liver injury. As shown in Fig. 3.4, the successful induction of Con-A induced hepatitis was demonstrated by increased serum AST and ALT levels, and necrosis and degeneration of hepatic cells in WT C57BL/6 mice. However, in consistence with the reduction of iVα14 NKT cells in B7- and CD28- deficient mice, serum AST and ALT levels were markedly reduced more than 2-fold in mutant mice as compared with WT mice (Fig. 3.3A). Although the pathological patterns were the same in three groups, the severities of liver injuries were decreased 4-5 fold in B7- and CD28- deficient mice (Fig. 3.4, B and c), demonstrated by the percentage of injured areas on whole area of liver sections. There is no difference

between B7 and CD28 deficient mice. Therefore, the partial resistance to Con-A induced hepatitis were mainly due to the functional defect of iVα14 NKT cells in B7- and CD28-deficient mice.

## Discussion

The prerequisite of thymus in the development of NKT has been demonstrated by the severe deficiency of peripheral NKT cells resulting from thymectomic mice (36) and mature NKT cell generated in liver from thymus-grafted mice (37). This indicates the spatial relationship between conventional T cell and NKT cell development. Moreover, the disruption of both conventional T cell and NKT cell development could be found in mice with deficiencies in recombinase machinery for antigen receptor gene rearrangement and most important, pre-Tα mice (12, 38), supported that conventional T cell and NKT cell may arise from the same precursor, or they share same signaling pathway in their developments. The direct evidence for this relationship was provided by Gapin et al. whose data showed that mature NKT cells can be developed from intrathymically injected $CD4^{+}CD8^{+}$ thymocytes (13). We previously investigated the critical role of costimulatory signaling in T cell development at DN stage, which showed that in addition to pre-TCR signaling, B7/CD28 costimulatory pathway, functions as the second signal in very early stage of T cell development (21). They play an important role by controlling immature DN cell proliferation and promoting the appropriate transition between sequential differential stages. If NKT cell shares the same progenitor with conventional T cell and deviated a distinct linage at DP stage in the thymus as recent publication suggested (11-13), it would be expected that the B7-CD28 signaling, which plays important role in early stage of conventional T cell development, would also be critical in controlling the development of NKT cells.

In our study, the $TCR^+NK1.1^+$ population is decreased 3-5 fold in thymus and about 2 fold in spleen of B7 and CD28 deficient mice. This prompted us to further examine this population using αGalCer-loaded CD1d tetramers that bind specifically to the invariant Vα14-Jα18 TCR (29, 33), the iVα14 NKT cells. Similarly, the percentage of $TCR^+$α-GalCer/Cd1d $^+$ population, the iVα14 NKT cells, is also reduced from total thymocytes and splenocytes. Although the reductions of NKT cells by both definitive markers are not detectable in livers from B71/2 and CD28 mutant mice compared with WT mice, the triple positive populations (α-GalCer/Cd1d $^+$ populations in $TCR^+NK1.1^+$ cells) are significantly decreased in liver lymphocytes. In consistency with the reduced numbers of iVα14 NKT cells in liver, the defective function of iVα14 NKT cells are also manifested by less severe liver injury in ConA induced hepatitis model, which had been proved directly mediated by this unique subset of lymphocytes (34, 35). Therefore, costimulatory pathway involves in the development and the function of iVα14 NKT cells.

The most interesting result from present studies is that the significant reduction of $NK1.1^+CD4^-$ subsets and increased $NK1.1^-CD4^+$ subsets in gated $TCR^+$ α-GalCer/Cd1d $^+$ iVα14 NKT cells in B7 and CD28 deficient mice. Similar patterns could be found not only in thymus but also for spleen and liver as well. The relationship of these two subsets is very controversial. It has been proposed that the $NK1.1^-CD4^+$ cells are pre-mature NKT cells at early developmental stage in the thymus (16, 17), and they are originally $CD44^{low}$ with a faster proliferation rate. By intrathymic injection of sorted $NK1.1^-$α-GalCer/Cd1d $^-$ thymocytes into Jα18-/- recipients, Benlagha et al. demonstrated that these cells could express NK1.1 within a week from thymus, spleen and liver of recipients, which are originally lack endogenous iVα14 NKT precursors (16). This supports the

developmental relationship between $NK1.1^{-}CD4^{+}$ and $NK1.1^{+}CD4^{-}$ cells. Therefore, the developmental sequence has been suggested from $CD44^{low}NK1.1^{-}$ to $CD44^{high}NK1.1^{-}$ to $CD44^{high}NK1.1^{+}$ (16, 39), and other NK linage marker may even expressed later(13). Further more, the present of this immature $NK1.1^{-}CD4^{+}$ cells in the spleen and liver was explained by that the later maturation is not thymic independent (17). However, the co-existence of both $NK1.1^{-}CD4^{+}$ and $NK1.1^{+}CD4^{-}$ cells not only in peripheral lymphoid organs but also in thymus. Secondly, the expression level of CD44 in high in $NK1.1^{-}CD4^{+}$ subset, particularly the thymic recent emigrants (16) in the periphery are all $CD44^{+}$. Thirdly, $NK1.1^{-}CD4^{+}$ is able to produce cytokines when encountered with α-GalCer pulsed splenic DC (16, 17), and purified $NK1.1^{+}$ and $NK1.1^{-}$ NKT cell populations show similar TCR down-modulation kinetics and proliferation potential in response to α-GalCer stimulation in vitro (40). All these phenomena cannot be simply explained by clear-cut immature and mature phenotypes between $NK1.1^{-}CD4^{+}$ and $NK1.1^{+}CD4^{-}$ cells. On the other hand, even the phenotype regarding cell surface markers are same, peripheral $NK1.1^{-}CD4^{+}$ or $NK1.1^{+}CD4^{-}$ cells may not necessarily be the same maturation with their thymic analogues. It has been shown that peripheral $NK1.1^{+}$ NKT cells has a rapider turn over rate than thymic $NK1.1^{+}$ NKT cells (37). Taken together, these results indicate that there is functional distinction between thymic and peripheral $NK1.1^{-}CD4^{+}$ cells, in another word, at least the peripheral $NK1.1^{-}CD4^{+}$ may not completely functions as the precursor of peripheral $NK1.1^{+}CD4^{-}$ iVα14 NKT cells. The peripheral $NK1.1^{-}CD4^{+}$ iVα14 NKT cells, or part of them if not all, present as another functionally matured, fully differentiated subset of iVα14 NKT cells. The transition between $NK1.1^{-}$ and $NK1.1^{+}$ iVα14 NKT cells maybe due to the activation status as for CD8+ T cells (30), and

costimulation signaling could involves in this process. Therefore, the reduction of $NK1.1^-$ $CD4^+$ subset and increasing of $NK1.1^+CD4^-$ subset in B7 and CD28 deficient mice demonstrated that costimulatory pathway involved in optimal activation of iVα14 NKT cells, and play an important role in differentiation and reconstitution of iVα14 NKT subsets. We tested the constitution of iVα14 NKT as early as Day5 and Day 8 (data not shown), and we cannot see the difference between B7, CD28 deficient and WT mice. This indicated that the constitutional differences are not exclusively due to developmental defect on immature iVα14 NKT cells.

The even complicate constitution of α-GalCer restricted iVα14 NKT cells that includes different functional subsets had been proposed before (26, 41), they are defined by different phenotypes ($CD4^+$ vs. $CD4^-$, or $NK1.1^+$ vs. $NK1.1^-$) (41, 42) and different specific microenvironments (43). They also function differently by secreting different cytokine profiles when stimulated with α-GalCer. $Nk1.1^-$ iVα14 NKT cells primarily produce IL-4 (Th2), while $NK^+$ iVα14 NKT cells generated more IFN-γ (Th1) than IL-4 (16, 17, 42, 44). In ConA induced hepatitis model, Th2 type cytokine IL-4 secreted by iVα14 NKT cells, was demonstrated playing a crucial role to cause NKT cell express FasL and contribute to Fas/FasL-mediated liver injury in an autocrine fashion (34, 35, 45). Apparently, the changed proportion of $NK1.1^-CD4^+$ and $NK1.1^+CD4^-$ cells in B7 and CD28 deficient mice should prone to IL-4 production and hence, increase the susceptibility of liver injury. However, ConA induced hepatitis is a multiple-steps disease process. Th1 type cytokine IFN-γ also activates resident Kupffer cells and recruit macrophages to produce TNF-α, which subsequently causes liver injury (34). Moreover, other immunoregulatory cytokines, such as IL-5 (45, 46) and IL-6 (47), also had been

demonstrated to mediate the pathogenesis of ConA-induced hepatitis. Thus, the moderate elevate IL-4 won't exclusively enhance the liver injury. In addition, the reduced total iVα14 NKT cells number will lower the total IL-4 production and alleviate the ConA-induced liver injury.

Admittedly, NKT cells fail to develop in mice lacking lymphotoxin (LT) (48), or components of the IL-15 pathway, such as IL-15 and IL-15Rα, and IL-2/15Rβ (49) , whereas conventional T cell development in these mice is almost normal (5). However, these deficient mice demonstrated a relatively developmental defect on NK cell (48, 49). Therefore, in addition to the developmental pathway shared with conventional T cells, part of NKT developments could be shared with NK cells. NK cells are developed in bone marrow instead of thymus, and NK cells exported out of BM are already matured and could be found in peripheral blood, spleen, liver and as well as uterus (50). Moreover, Townsend et al had demonstrated that mice with a targeted deletion of T-bet, a T-box transcription factor required for Th1 cell differentiation, have a profound, stem cell-intrinsic defect in their ability to generate mature NK and Valpha14i NKT cells. Both cell types fail to complete normal terminal maturation and are present in decreased numbers in peripheral lymphoid organs of T-bet(-/-) mice (51). Therefore, these data further demonstrated that there are some common developmental molecular pathway during the final maturation stages of both NK and iVα14 NKT cells. Interestingly, in our studies, we also found reduction of NK cells, in term of NK1.1+TCRβ-, in the spleen and liver of B7- and CD28-deficient mice. It has been shown costimulatory molecules are necessary for optimal NK cell functions, but their function on NK development have never been

reported before. Whether these costimulatory molecules represent important signaling to NK development needs to be further determined. The decreased NK cell population may also contribute to alleviate ConA induced hepatitis in mutant mice (52).

In conclusion, the present study expands the functions of costimulatory molecules from T cell to NKT cells. B7 and CD28 interaction is critical to the differentiation and functional competence of iVα14 NKT cells. In addition, these data highlight the points that developing NKT cells end up to phenotypically distinct subsets and is characterized by a dynamic process involving up- and down-regulation of CD4 and NK1.1. Costimulatory molecules involve in this process by keeping the optimal activation of NKT cells and modulating the proportion of different subsets. Further understanding and elucidating the mechanisms of this modulation may lead to the discovery and use of new therapeutic agents targeting at expansion of specific subset of iVα14 NKT cells.

**Reference**


1. Taniguchi, M., K. Seino, and T. Nakayama. 2003. The NKT cell system: bridging innate and acquired immunity. *Nat Immunol* 4:1164-1165.
2. Taniguchi, M., M. Harada, S. Kojo, T. Nakayama, and H. Wakao. 2003. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu Rev Immunol* 21:483-513.
3. Bendelac, A., O. Lantz, M.E. Quimby, J.W. Yewdell, J.R. Bennink, and R.R. Brutkiewicz. 1995. CD1 recognition by mouse NK1+ T lymphocytes. *Science* 268:863-865.
4. Bendelac, A., M.N. Rivera, S.H. Park, and J.H. Roark. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu Rev Immunol* 15:535-562.
5. Kronenberg, M., and L. Gapin. 2002. The unconventional lifestyle of NKT cells. *Nat Rev Immunol* 2:557-568.
6. Matsuda, J.L., L. Gapin, J.L. Baron, S. Sidobre, D.B. Stetson, M. Mohrs, R.M. Locksley, and M. Kronenberg. 2003. Mouse V alpha 14i natural killer T cells are resistant to cytokine polarization in vivo. *Proc Natl Acad Sci U S A* 100:8395-8400.
7. Stetson, D.B., M. Mohrs, R.L. Reinhardt, J.L. Baron, Z.E. Wang, L. Gapin, M. Kronenberg, and R.M. Locksley. 2003. Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J Exp Med* 198:1069-1076.

8. Van Dommelen, S.L., and M.A. Degli-Esposti. 2004. NKT cells and viral immunity. *Immunol Cell Biol* 82:332-341.

9. Swann, J., N.Y. Crowe, Y. Hayakawa, D.I. Godfrey, and M.J. Smyth. 2004. Regulation of antitumour immunity by CD1d-restricted NKT cells. *Immunol Cell Biol* 82:323-331.

10. Van Kaer, L. 2004. Natural killer T cells as targets for immunotherapy of autoimmune diseases. *Immunol Cell Biol* 82:315-322.

11. MacDonald, H.R. 2002. Immunology. T before NK. *Science* 296:481-482.

12. MacDonald, H.R. 2002. Development and selection of NKT cells. *Curr Opin Immunol* 14:250-254.

13. Gapin, L., J.L. Matsuda, C.D. Surh, and M. Kronenberg. 2001. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. *Nat Immunol* 2:971-978.

14. Chun, T., M.J. Page, L. Gapin, J.L. Matsuda, H. Xu, H. Nguyen, H.S. Kang, A.K. Stanic, S. Joyce, W.A. Koltun, M.J. Chorney, M. Kronenberg, and C.R. Wang. 2003. CD1d-expressing dendritic cells but not thymic epithelial cells can mediate negative selection of NKT cells. *J Exp Med* 197:907-918.

15. Pellicci, D.G., A.P. Uldrich, K. Kyparissoudis, N.Y. Crowe, A.G. Brooks, K.J. Hammond, S. Sidobre, M. Kronenberg, M.J. Smyth, and D.I. Godfrey. 2003. Intrathymic NKT cell development is blocked by the presence of alpha-galactosylceramide. *Eur J Immunol* 33:1816-1823.

16. Benlagha, K., T. Kyin, A. Beavis, L. Teyton, and A. Bendelac. 2002. A thymic precursor to the NK T cell lineage. *Science* 296:553-555.

17. Pellicci, D.G., K.J. Hammond, A.P. Uldrich, A.G. Baxter, M.J. Smyth, and D.I. Godfrey. 2002. A natural killer T (NKT) cell developmental pathway iInvolving a thymus-dependent NK1.1(-)CD4(+) CD1d-dependent precursor stage. *J Exp Med* 195:835-844.

18. Hare, K.J., J. Pongracz, E.J. Jenkinson, and G. Anderson. 2003. Modeling TCR signaling complex formation in positive selection. *J Immunol* 171:2825-2831.

19. Amsen, D., and A.M. Kruisbeek. 1996. CD28-B7 interactions function to co-stimulate clonal deletion of double-positive thymocytes. *Int Immunol* 8:1927-1936.

20. Gao, W., S. Kumar, M.T. Lotze, C. Hanning, P.D. Robbins, and A. Gambotto. 2003. Innate immunity mediated by the cytokine IL-1 homologue 4 (IL-1H4/IL-1F7) induces IL-12-dependent adaptive and profound antitumor immunity. *J Immunol* 170:107-113.

21. Zheng, X., J.X. Gao, X. Chang, Y. Wang, Y. Liu, J. Wen, H. Zhang, J. Zhang, and P. Zheng. 2004. B7-CD28 interaction promotes proliferation and survival but suppresses differentiation of CD4-CD8- T cells in the thymus. *J Immunol* 173:2253-2261.

22. Emoto, M., and S.H. Kaufmann. 2003. Liver NKT cells: an account of heterogeneity. *Trends Immunol* 24:364-369.

23. Crispe, I.N. 2003. Hepatic T cells and liver tolerance. *Nat Rev Immunol* 3:51-62.

24. Exley, M.A., and M.J. Koziel. 2004. To be or not to be NKT: natural killer T cells in the liver. *Hepatology* 40:1033-1040.

25. Eberl, G., R. Lees, S.T. Smiley, M. Taniguchi, M.J. Grusby, and H.R. MacDonald. 1999. Tissue-specific segregation of CD1d-dependent and CD1d-independent NK T cells. *J Immunol* 162:6410-6419.

26. Hammond, K.J., S.B. Pelikan, N.Y. Crowe, E. Randle-Barrett, T. Nakayama, M. Taniguchi, M.J. Smyth, I.R. van Driel, R. Scollay, A.G. Baxter, and D.I. Godfrey. 1999. NKT cells are phenotypically and functionally diverse. *Eur J Immunol* 29:3768-3781.

27. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki, and M. Taniguchi. 1997. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278:1626-1629.

28. Benlagha, K., A. Weiss, A. Beavis, L. Teyton, and A. Bendelac. 2000. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. *J Exp Med* 191:1895-1903.

29. Matsuda, J.L., O.V. Naidenko, L. Gapin, T. Nakayama, M. Taniguchi, C.R. Wang, Y. Koezuka, and M. Kronenberg. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med* 192:741-754.

30. Assarsson, E., T. Kambayashi, J.K. Sandberg, S. Hong, M. Taniguchi, L. Van Kaer, H.G. Ljunggren, and B.J. Chambers. 2000. CD8+ T cells rapidly acquire NK1.1 and NK cell-associated molecules upon stimulation in vitro and in vivo. *J Immunol* 165:3673-3679.

31. Slifka, M.K., R.R. Pagarigan, and J.L. Whitton. 2000. NK markers are expressed on a high percentage of virus-specific CD8+ and CD4+ T cells. *J Immunol* 164:2009-2015.

32. Hammond, K.J., D.G. Pellicci, L.D. Poulton, O.V. Naidenko, A.A. Scalzo, A.G. Baxter, and D.I. Godfrey. 2001. CD1d-restricted NKT cells: an interstrain comparison. *J Immunol* 167:1164-1173.

33. Sidobre, S., and M. Kronenberg. 2002. CD1 tetramers: a powerful tool for the analysis of glycolipid-reactive T cells. *J Immunol Methods* 268:107-121.

34. Takeda, K., Y. Hayakawa, L. Van Kaer, H. Matsuda, H. Yagita, and K. Okumura. 2000. Critical contribution of liver natural killer T cells to a murine model of hepatitis. *Proc Natl Acad Sci U S A* 97:5498-5503.

35. Kaneko, Y., M. Harada, T. Kawano, M. Yamashita, Y. Shibata, F. Gejyo, T. Nakayama, and M. Taniguchi. 2000. Augmentation of Valpha14 NKT cell-mediated cytotoxicity by interleukin 4 in an autocrine mechanism resulting in the development of concanavalin A-induced hepatitis. *J Exp Med* 191:105-114.

36. Hammond, K., W. Cain, I. van Driel, and D. Godfrey. 1998. Three day neonatal thymectomy selectively depletes NK1.1+ T cells. *Int Immunol* 10:1491-1499.

37. Coles, M.C., and D.H. Raulet. 2000. NK1.1+ T cells in the liver arise in the thymus and are selected by interactions with class I molecules on CD4+CD8+ cells. *J Immunol* 164:2412-2418.

38. Eberl, G., H.J. Fehling, H. von Boehmer, and H.R. MacDonald. 1999. Absolute requirement for the pre-T cell receptor alpha chain during NK1.1+ TCRalphabeta cell development. *Eur J Immunol* 29:1966-1971.

39. Berzins, S.P., A.P. Uldrich, D.G. Pellicci, F. McNab, Y. Hayakawa, M.J. Smyth, and D.I. Godfrey. 2004. Parallels and distinctions between T and NKT cell development in the thymus. *Immunol Cell Biol* 82:269-275.

40. Wilson, M.T., C. Johansson, D. Olivares-Villagomez, A.K. Singh, A.K. Stanic, C.R. Wang, S. Joyce, M.J. Wick, and L. Van Kaer. 2003. The response of natural killer T cells to glycolipid antigens is characterized by surface receptor down-modulation and expansion. *Proc Natl Acad Sci U S A* 100:10913-10918.

41. Godfrey, D.I., H.R. MacDonald, M. Kronenberg, M.J. Smyth, and L. Van Kaer. 2004. NKT cells: what's in a name? *Nat Rev Immunol* 4:231-237.

42. Stenstrom, M., M. Skold, A. Ericsson, L. Beaudoin, S. Sidobre, M. Kronenberg, A. Lehuen, and S. Cardell. 2004. Surface receptors identify mouse NK1.1+ T cell subsets distinguished by function and T cell receptor type. *Eur J Immunol* 34:56-65.

43. Yang, Y., A. Ueno, M. Bao, Z. Wang, J.S. Im, S. Porcelli, and J.W. Yoon. 2003. Control of NKT cell differentiation by tissue-specific microenvironments. *J Immunol* 171:5913-5920.

44. Loza, M.J., L.S. Metelitsa, and B. Perussia. 2002. NKT and T cells: coordinate regulation of NK-like phenotype and cytokine production. Eur J Immunol 32:3453-3462.

45. Jaruga, B., F. Hong, R. Sun, S. Radaeva, and B. Gao. 2003. Crucial role of IL-4/STAT6 in T cell-mediated hepatitis: up-regulating eotaxins and IL-5 and recruiting leukocytes. J Immunol 171:3233-3244.

46. Louis, H., A. Le Moine, V. Flamand, N. Nagy, E. Quertinmont, F. Paulart, D. Abramowicz, O. Le Moine, M. Goldman, and J. Deviere. 2002. Critical role of interleukin 5 and eosinophils in concanavalin A-induced hepatitis in mice. Gastroenterology 122:2001-2010.

47. Sun, R., Z. Tian, S. Kulkarni, and B. Gao. 2004. IL-6 prevents T cell-mediated hepatitis via inhibition of NKT cells in CD4+ T cell- and STAT3-dependent manners. J Immunol 172:5648-5655.

48. Iizuka, K., D.D. Chaplin, Y. Wang, Q. Wu, L.E. Pegg, W.M. Yokoyama, and Y.X. Fu. 1999. Requirement for membrane lymphotoxin in natural killer cell development. Proc Natl Acad Sci U S A 96:6336-6340.

49. Kennedy, M.K., M. Glaccum, S.N. Brown, E.A. Butz, J.L. Viney, M. Embers, N. Matsuki, K. Charrier, L. Sedger, C.R. Willis, K. Brasel, P.J. Morrissey, K. Stocking, J.C.L. Schuh, S. Joyce, and J.J. Peschon. 2000. Reversible Defects in Natural Killer and Memory CD8 T Cell Lineages in Interleukin 15-deficient Mice. J. Exp. Med. 191:771-780.

50. Yokoyama, W.M., S. Kim, and A.R. French. 2004. The dynamic life of natural killer cells. Annu Rev Immunol 22:405-429.

51. Townsend, M.J., A.S. Weinmann, J.L. Matsuda, R. Salomon, P.J. Farnham, C.A. Biron, L. Gapin, and L.H. Glimcher. 2004. T-bet regulates the terminal maturation and homeostasis of NK and Valpha14i NKT cells. Immunity 20:477-494.

52. Trobonjaca, Z., A. Kroger, D. Stober, F. Leithauser, P. Moller, H. Hauser, R. Schirmbeck, and J. Reimann. 2002. Activating immunity in the liver. II. IFN-beta

attenuates NK cell-dependent liver injury triggered by liver NKT cell activation. J Immunol 168:3763-3770.

**Figure legends.**

**Figure 1. The reduced TCR $\beta^+NK1.1^+$ NKT cells in the thymus, spleen and liver of B7-1/2 and CD28 deficient mice.** B7KO, CD28KO and C57BL/6 mice were sacrificed at 8 weeks old. Total lymphocytes (left column) from viable cells, $CD4^-CD8^-$ DN lymphocytes (middle column) and $CD4^+$ lymphocytes (right column) were gated and look at the distribution of TCRβ and Nk1.1. The NKT cell was denoted by TCR $\beta^+NK1.1^+$ and NK cell in the spleen and liver was denoted by TCR $\beta^-NK1.1^+$. The numbers are represented as percentages (Mean±SD) of gated NKT or NK cells. A. In thyomcytes. TCR $\beta^+NK1.1^+$ cells are decreased almost 3-5 folds in total thymocytes, DN and CD4+ thymocytes as well. The mutation of CD28 demonstrated even more significant effect on total and $CD4^+$, but not DN thymocytes. B. In splenocytes. TCR $\beta^+NK1.1^+$ cells were decreased in total and $CD4^+$ splenocytes, but not on DN splenocytes, from both genetic mutant mice. The NK cells are also reduced about 30% in total and DN splenocytes. C. In liver lymphocytes. The reductions of TCR $\beta^+NK1.1^+$ cells are not significant except in $CD4^+$ lymphocytes in CD28 mutant mice. But the reduction of NK cells is consistent with that in spleen. P value indicates the statistic difference between compared groups. One representative from each group (totally 9 in each group from 2 separate experiments) was shown here.

**Figure 2. The significant reduction of CD1d restricted TCR $\beta^+NK1.1^+$ cells in B7 and CD28 mutant mice.** TCR$\beta^+NK1.1^+$ cells were gated from total lymphocytes from thymus (left column), spleen (middle column) and liver (right column) of 8 wks old B7KO, CD28KO and C57BL/6 mice, then were stained with CD44 and α-GalCer/CD1d tetramer. The number represented the percentage (mean±SD) of α-GalCer/$CD1d^+CD44^+$

cells (CD1d restricted iVα14 NKT cells) in TCR $\beta^+NK1.1^+$ population. P value indicates the statistic difference between compared two groups. One representative from each group (totally 9 in each group from 2 separate experiments) was shown here.

**Figure 3. The reduction of α-GalCer/CD1d$^+$TCRβ$^+$ NKT cells in B7 and CD28 mutant mice.** TCR$\beta^+NK1.1^+$ cells were gated from total lymphocytes from thymus (left column), spleen (middle column) and liver (right column) of 8 wks old B7KO, CD28KO and C57BL/6 mice, then were stained with CD44 and α-GalCer/CD1d tetramer. The number represented the percentage (mean±SD) of α-GalCer/CD1d$^+$CD44$^+$ cells (CD1d restricted iVα14 NKT cells) in TCR $\beta^+NK1.1^+$ population. P value indicates the statistic difference between compared two groups. One representative from each group (totally 9 in each group from 2 separate experiments) was shown here.

**Figure 4. Con A induced hepatitis is alleviated in B7 and CD28 deficient mice.** 8 wks old B7KO, CD28KO and C57BL/6 mice were i.v. injected with Con A at a dose of 20mg/kg. 16 hrs later, Sera and liver from individual mice were obtained. Serum aminotransferase [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)] activities were measured and histological examination was performed. a. Serum ALT and AST level. b. One representative from each group showing the pattern of liver injury, which indicated the typical degeneration and apoptosis of hepatocytes. The histological changes are the same within three groups. c. Percentage of injury area from total area on each section. 3-4 sections were collected from each mouse, totally 4-5 mice in each group. *P<005, ** P<0.01, ***P<0.001.

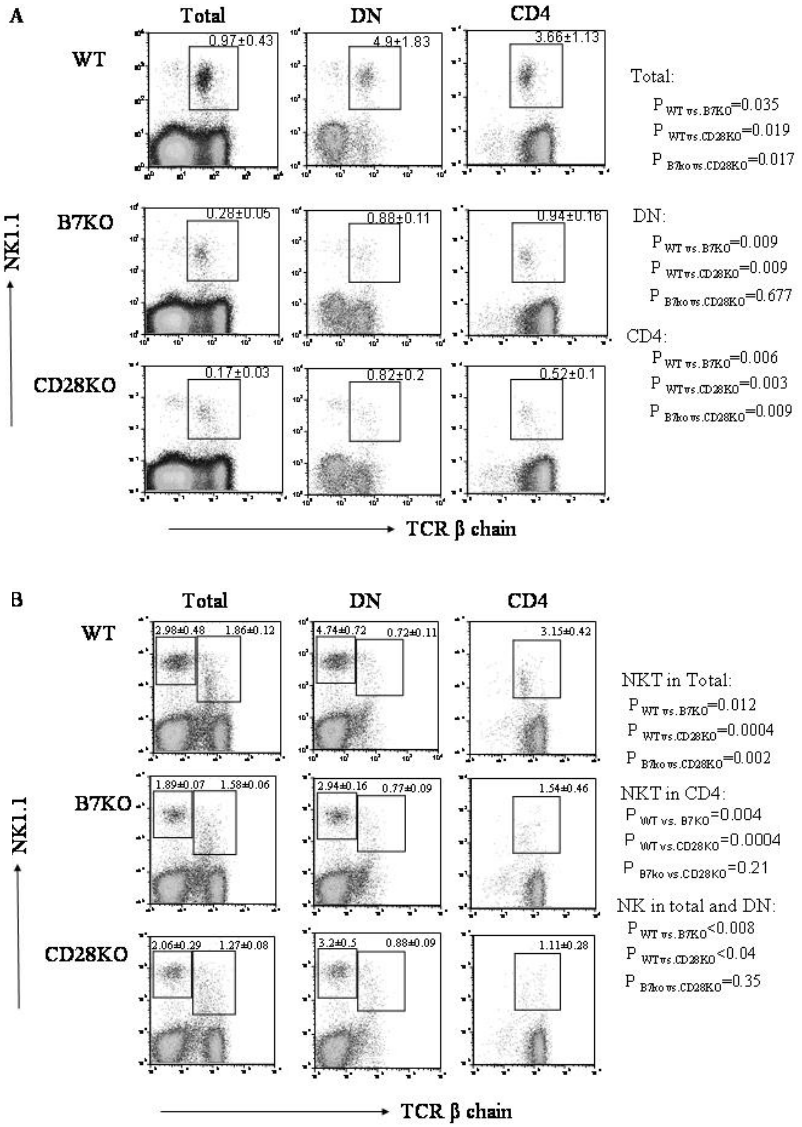
A
Total
DN
CD4
WT
B7KO
CD28KO
NK1.1
TCR β chain
0.97±0.43
4.9±1.83
3.66±1.13
0.28±0.05
0.88±0.11
0.94±0.16
0.17±0.03
0.82±0.2
0.52±0.1
Total:
P WT vs.B7KO=0.035
P WTvs.CD28KO=0.019
P B7ko vs.CD28KO=0.017
DN:
P WT vs.B7KO=0.009
P WTvs.CD28KO=0.009
P B7ko vs.CD28KO=0.677
CD4:
P WT vs.B7KO=0.006
P WTvs.CD28KO=0.003
P B7ko vs.CD28KO=0.009
B
Total
DN
CD4
WT
B7KO
CD28KO
NK1.1
TCR β chain
2.98±0.48
1.86±0.12
4.74±0.72
0.72±0.11
3.15±0.42
1.89±0.07
1.58±0.06
2.94±0.16
0.77±0.09
1.54±0.46
2.06±0.29
1.27±0.08
3.2±0.5
0.88±0.09
1.11±0.28
NKT in Total:
P WT vs.B7KO=0.012
P WTvs.CD28KO=0.0004
P B7ko vs.CD28KO=0.002
NKT in CD4:
P WT vs. B7KO=0.004
P WT vs.CD28KO=0.0004
P B7ko vs.CD28KO=0.21
NK in total and DN:
P WT vs.B7KO<0.008
P WTvs.CD28KO<0.04
P B7ko vs.CD28KO=0.35

**Fig. 1.**

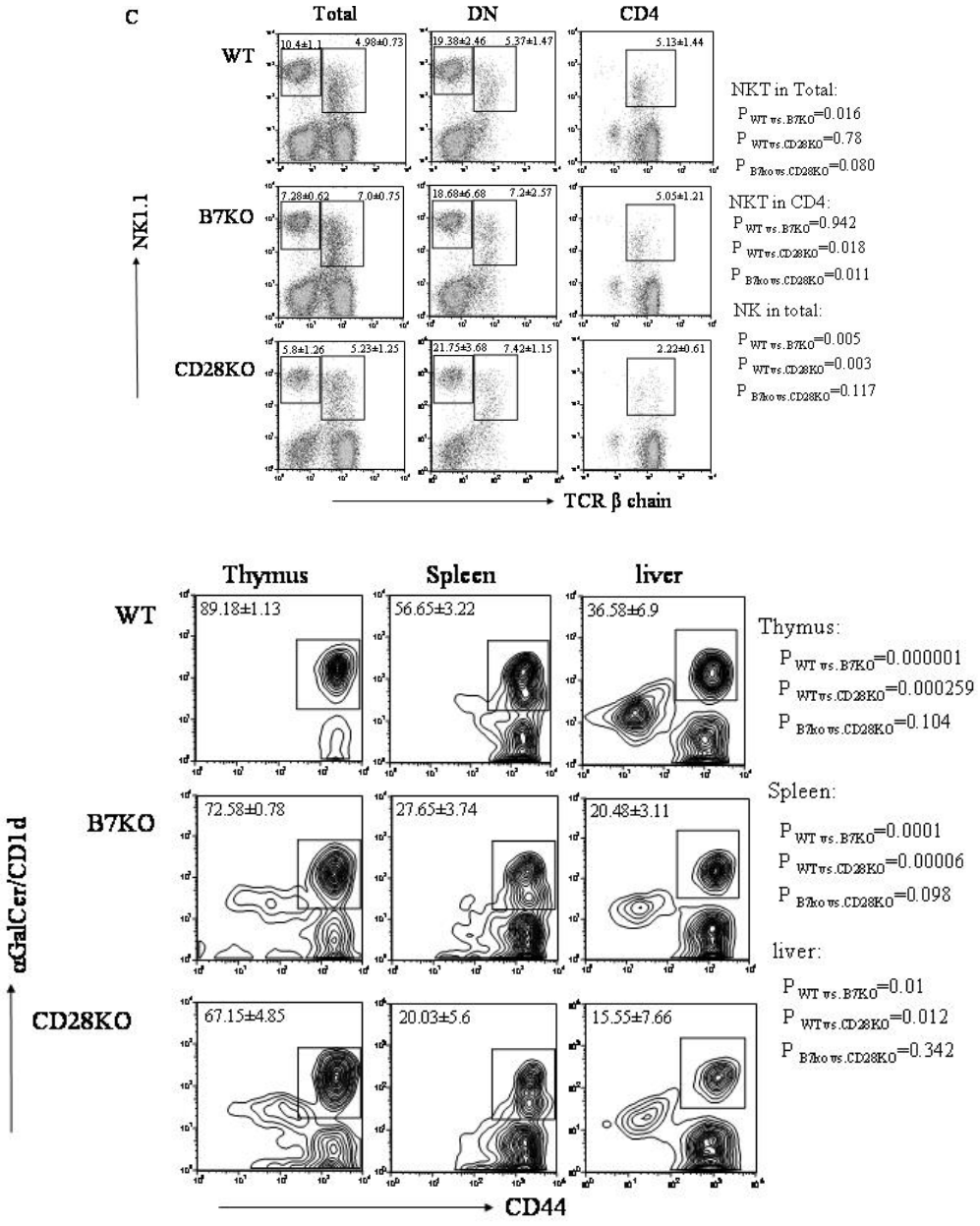
C
Total
DN
CD4
WT
B7KO
CD28KO
NK1.1
TCR β chain
10.4±1.1
4.98±0.73
19.38±2.46
5.37±1.47
5.13±1.44
7.28±0.62
7.0±0.75
18.68±6.68
7.2±2.57
5.05±1.21
5.8±1.26
5.23±1.25
21.75±3.68
7.42±1.15
2.22±0.61
NKT in Total:
P WT vs.B7KO=0.016
P WTvs.CD28KO=0.78
P B7ko vs.CD28KO=0.080
NKT in CD4:
P WT vs.B7KO=0.942
P WTvs.CD28KO=0.018
P B7ko vs.CD28KO=0.011
NK in total:
P WT vs.B7KO=0.005
P WTvs.CD28KO=0.003
P B7ko vs.CD28KO=0.117
Thymus
Spleen
liver
WT
B7KO
CD28KO
αGalCer/CD1d
CD44
89.18±1.13
56.65±3.22
36.58±6.9
72.58±0.78
27.65±3.74
20.48±3.11
67.15±4.85
20.03±5.6
15.55±7.66
Thymus:
P WT vs.B7KO=0.000001
P WTvs.CD28KO=0.000259
P B7ko vs.CD28KO=0.104
Spleen:
P WT vs.B7KO=0.0001
P WTvs.CD28KO=0.00006
P B7ko vs.CD28KO=0.098
liver:
P WT vs.B7KO=0.01
P WTvs.CD28KO=0.012
P B7ko vs.CD28KO=0.342

**Fig. 2.**

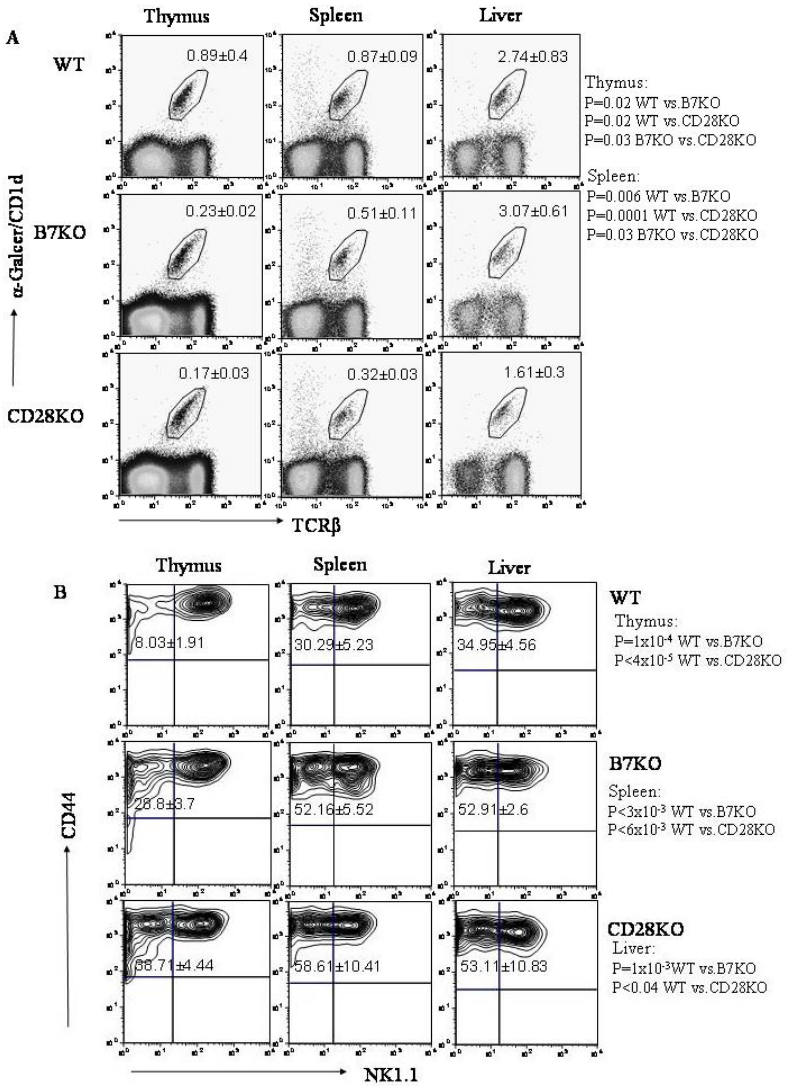
A
Thymus
Spleen
Liver
WT
B7KO
CD28KO
α-Galcer/CD1d
TCRβ
0.89±0.4
0.87±0.09
2.74±0.83
0.23±0.02
0.51±0.11
3.07±0.61
0.17±0.03
0.32±0.03
1.61±0.3
Thymus:
P=0.02 WT vs.B7KO
P=0.02 WT vs.CD28KO
P=0.03 B7KO vs.CD28KO
Spleen:
P=0.006 WT vs.B7KO
P=0.0001 WT vs.CD28KO
P=0.03 B7KO vs.CD28KO
B
Thymus
Spleen
Liver
CD44
NK1.1
8.03±1.91
30.29±5.23
34.95±4.56
28.8±3.7
52.16±5.52
52.91±2.6
38.71±4.44
58.61±10.41
53.11±10.83
WT
Thymus:
P=1x10-4 WT vs.B7KO
P<4x10-5 WT vs.CD28KO
B7KO
Spleen:
P<3x10-3 WT vs.B7KO
P<6x10-3 WT vs.CD28KO
CD28KO
Liver:
P=1x10-3WT vs.B7KO
P<0.04 WT vs.CD28KO

**Fig. 3-1.**

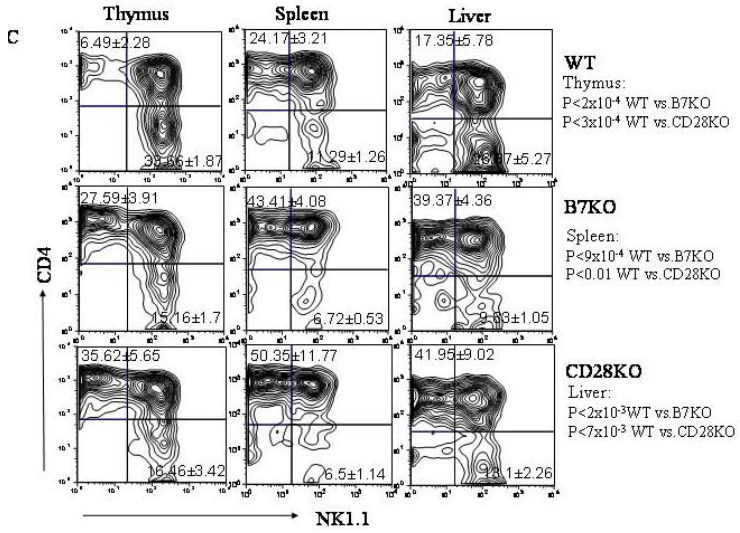
C
Thymus
Spleen
Liver
6.49±2.28
24.17±3.21
17.35±5.78
WT
Thymus:
P<2x10-4 WT vs.B7KO
P<3x10-4 WT vs.CD28KO
27.59±3.91
43.41±4.08
39.37±4.36
B7KO
Spleen:
P<9x10-4 WT vs.B7KO
P<0.01 WT vs.CD28KO
35.62±5.65
50.35±11.77
41.95±9.02
CD28KO
Liver:
P<2x10-3WT vs.B7KO
P<7x10-3 WT vs.CD28KO
CD4
NK1.1

**Fig. 3-2**

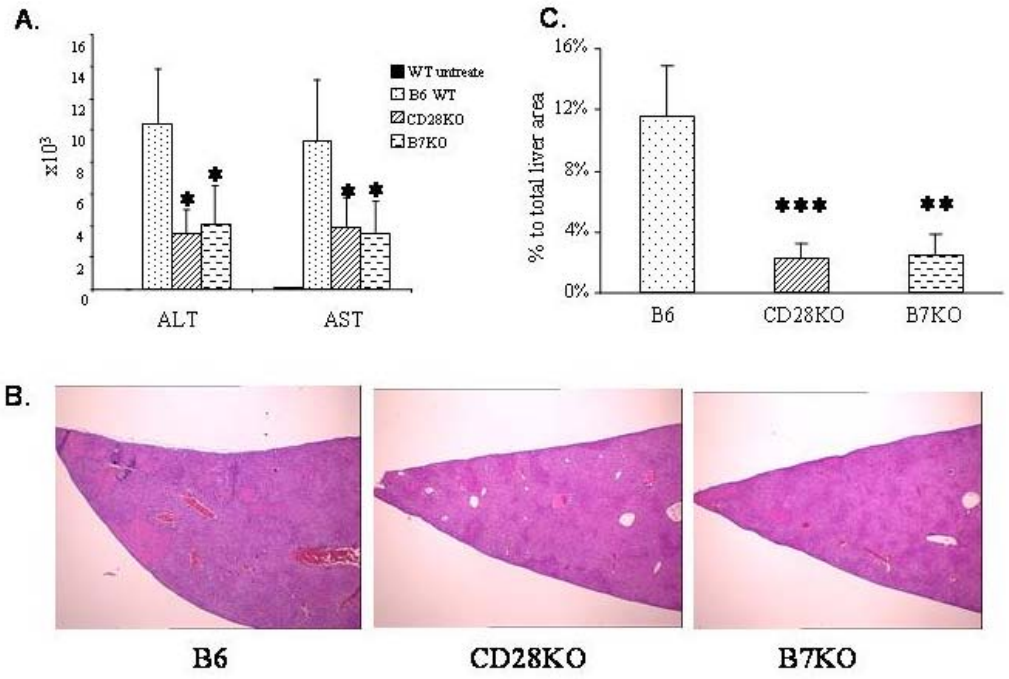
A.
x10³
WT untreate
B6 WT
CD28KO
B7KO
ALT
AST
C.
% to total liver area
16%
12%
8%
4%
0%
***
**
B6
CD28KO
B7KO
B.
B6
CD28KO
B7KO

**Fig. 4.**